Bacterial endophytes of rice

Hardoim, Pablo Rodrigo

Bacterial Endophytes of Rice – Their Diversity, Characteristics and Perspectives

Pablo Rodrigo Hardoim 2011

This study was supported by the joint RUG-WUR initiative on rice endophytes in the context of a DOE-JGI project on the rice endophyte metagenome.
This PhD project was carried out at the Departament of Microbial Ecology, according to the requirements of the Graduate School of Science (Faculty of Mathematics and Natural Sciences).
Centre for Ecological and Evolutionary Studies,
University of Groningen, PO BOX 11103
9700 CC Groningen, The Netherlands
Plant Research International
Droevendaalsesteeg 1
6708PB Wageningen, The Netherlands

Cover design: Jolanda Brons
With summaries in English, Dutch and Portuguese

ISBN: 978-90-367-5085-1
ISBN: 978-90-367-5086-8 (digital)

The author gratefully acknowledges the financial support from the University of Groningen and the Faculty of Mathematics and Natural Sciences for printing of this thesis manuscript.

Printed and bound in Netherlands
on acid-free paper by Ridderprint BV, Ridderkerk

RIJKSUNIVERSITEIT GRONINGEN

Bacterial endophytes of rice – their diversity, characteristics and perspectives

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 14 oktober 2011
om 11:00 uur

door

Pablo Rodrigo Hardoim

geboren op 30 juni 1978

te Várzea Grande, Brazil

Promotor:	Prof. dr. ir. J.D. van Elsas
Copromotor:	Dr. L.S. van Overbeek
Beoordelingscommissie:	Prof. dr. H. Olff Prof. dr. J.A. van Veen Prof. dr. J.T.M. Elzenga

To my wife

Contents

Glossary

Bacterial endophyte: ('endo' = inside; 'phyte' = plant): a bacterium that occurs inside a plant. In practical terms, a bacterium that can be isolated from surface-sterilized plant tissue (Perotti, 1926; Henning & Villforth, 1940).

Colonization trait: bacterial trait that is involved in the plant colonization process.

Competent endophyte: a microorganism that successfully colonizes a plant by actively entering plant tissue and that has the capacity to tinker with plant physiology and be selectively favoured, leading to beneficial maintenance of the plant-microbe association.

Endosphere: the microenvironment inside the plant (between plant cells) that is colonized by microorganisms. The endosphere consists of the endorhizosphere (internal root tissue) and the endophyllosphere (internal shoot and leaf tissue).

Exosphere: the environment outside the plant, which might or might not be directly influenced by the plant. This includes the rhizosphere, the rhizoplane and the phyllosphere.

Facultative endophyte: an organism that can (optionally) live inside plants and in other habitats.

Obligate endophyte: an organism that is strictly bound to life inside a plant during its entire lifespan and that does not posses life stages outside the plant, except for plant-to-plant and plant-to-insect-to-plant transmission.

Opportunistic endophyte: an organism that occasionally enters plants and profits from the plant internal environment (nutrient availability, protection, and lack of competition) (Reinhold-Hurek & Hurek, 1998).

Passenger endophyte: an endophyte that enters the plant by accident in the absence of selective forces maintaining it in the plant internal tissue.

Phytosphere: the area influenced by plants.

Rhizobacteria: soil bacteria that live under direct influence of plant roots. The term is often used for plant growth promoting rhizobacteria (PGPR), referring to beneficial plant-microbe interactions.

Rhizoplane: the surface of plant roots.

Rhizosphere: the narrow layer of soil in the vicinity of plant roots that is directly influenced by the root (Hiltner, 1904).

List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABA	abscisic acid
ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1-carboxylate
ACO	1-aminocyclopropane-1-carboxylate oxydase
ACS	1-aminocyclopropane-1-carboxylate synthase
AHL	*N*-acyl homoserine lactone
AI-2	autoinducer-2 system
AOB	ammonia-oxidizing bacteria
BID	bacterial inoculation densities
BNF	biological nitrogen fixation
CAS	Chrome Azurol S
CFU	colony-forming unity
CKs	cytokinins
COGs	cluster of orthologous groups of proteins
DGGE	denaturing gradient gel electrophoresis
DSF	diffusible signal factor
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharides
ET	ethylene
FPTs	fingerprint types
FW	fresh weight
GAs	gibberellins
GFP	green fluorescent protein
GST	glutathione-S-transferases
HMW	high-molecular-weight
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
IMG	Integrated Microbial Genomes
IPyA	indole-3-pyruvate
IRRI	International Rice Research Institute
ISR	induced systemic resistance
JA	jasmonic acid
K	Kollumerwaard soil
LMW	low-molecular-weight
LPS	lipopolysaccharides
M	molar
MOMP	major outer membrane protein
NCBI	National Center for Biotechnology Information
NPT	new plant type
NUE	nitrogen use efficiency
OTUs	operational taxonomic units

PBS	phosphate buffered saline
PCA	principal components analysis
PCR	polymerase chain reaction
PCR-DGGE	PCR-based denaturing gradient gel electrophoresis
PGP	plant growth-promoting
PGPB	plant growth-promoting bacteria
PGPR	plant growth-promoting rhizobacteria
PHA	polyhydroxyalkanoates
PHB	polyhydroxybutyrates
PR	pathogenesis-related
PSB	phosphate-solubilizing bacteria
QS	quorum-sensing
RDA	redundancy analysis
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcription
RUBISCO	ribulose-1,5-bisphosphate carboxylase oxygenase
SA	salicylic acid
SAM	*S*-adenosyl methionine
SAR	systemic acquired resistance
T3SS	type III secretion system
T6SS	type VI secretion systems
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
T-RFLP	terminal restriction fragment length polymorphism
UV	ultraviolet
V	Valthermond soil
VOCs	volatile organic compounds
WHC	water-holding capacity

Chapter 1

Ecology of Bacterial Endophytes in Sustainable Agriculture[1]

Pablo R. Hardoim, Riitta Nissinen[2], and Jan D. van Elsas

Abstract

Plants are the major source of carbohydrates for the heterotrophic microorganisms on Earth. For their growth, the latter organisms thus rely heavily on the efficient production of photoassimilates by plants. Plants even make use of diverse compounds to interact, and form associations, with often mutualistic beneficial bacteria. On the other hand, bacteria possess a wide range of metabolic properties that may modulate plant growth. Bacteria living inside plants, i.e. bacterial endophytes, might intimately interact with cells of the host, taking up secreted metabolites and releasing plant-growth-promoting (PGP) compounds. This synergistic interaction has been recently demonstrated and exemplifies a so-called double-fitness trait which is active in the plant-endophyte partnership. The ecological role of bacterial endophytes that can improve sustainable agriculture is further discussed.

[1] Submitted as a chapter of the book *Bacteria in Agrobiology*, Springer; Maheshwari, D.K. (ed.). The original publication is available at www.springerlink.com

[2] The authors Hardoim and Nissinen contributed equaly to this manuscript

Introduction

Plants are autotrophic organisms capable of transforming light energy into chemical (carbonaceous) compounds. These photo-assimilated compounds, when secreted from plant roots, attract a variety of microorganisms that can directly affect the growth and development of the host plant. Plant roots secrete low-molecular-weight (LMW) compounds (e.g. organic acids, amino acids and sugars) next to high-molecular-weight (HMW) ones (e.g. mucilage, proteins and sloughed-off plant cells). The release of these compounds has the putative function to eliminate waste products from internal metabolic processes and to facilitate plant growth, for instance in external lubrication and nutrient acquisition (Bais 2004). Furthermore, the compounds in root exudates may affect biological processes through the regulation of mutualistic associations with neighbouring (micro)organisms. In this, beneficial interactions may be stimulated, whereas detrimental ones are antagonized (Bais 2006). Two relatively well-documented types of mutualistic interactions (i.e. those with beneficial rhizobia and tumor-inducing *Agrobacterium*) exemplify the importance of plant root exudates for the initiation of the interactions.

Until recently, plant roots have been considered as representing merely supporting plant tissues, which have the ability to absorb water and nutrients for plants to grow. However, with the increasing appreciation of how root exudates select specific soil microorganisms to interact and improve plant health (Hartmann et al. 2009), new investigations into the mechanisms involved in plant-bacterial interactions are flowing. By the process of root exudation, a rich source of 'readily-available' (e.g. LMW compounds) and recalcitrant nutrients diffuses into the rhizosphere (the soil which is directly affected by plant roots), attracting diverse heterotrophic microorganisms. The latter first colonize the rhizoplane (the surface of plant roots) and, later, a selected fraction of these may occupy the internal root tissues to become endophytic. Hence, most bacterial colonization traits that are observed in rhizobacteria are expected to be present in endosphere (**Chapter 2**, Hardoim et al. 2008). Furthermore, bacteria equipped with traits for efficient substrate acquisition, versatile nutrient metabolism, stress resistance and competitiveness might be at an advantage to become endophytic. In this respect, endophytes are those bacteria that occur inside a plant ('endo', inside; 'phyte', plant). In practical terms, it is often postulated that those bacteria that can be isolated from surface-sterilized plant tissues are endophytes. For the plant, common sources of bacterial endophytes are the soil surrounding roots (i.e. the exorhizosphere), the atmosphere (i.e. exophyllosphere) and vegetatively propagated plant material (e.g. seeds, stems and cuttings). Interestingly, multivariate analyses of

assigned COGs (cluster of orthologous groups of proteins) from selected metagenomes, including a rice metagenome, have revealed that bacterial endophytes indeed form a distinct community when compared to bacterial communities from soil or other environmental habitats (Fig. 1). The metabolic profile of the collective endophytes closely resembled that found in sludge systems and, surprisingly, differed from that of the communities sampled from soil or freshwater. This suggests that, although soil might indeed be the main source of bacterial endophytes, plants provide selective forces that favor communities that possess a distinct metabolic repertoire. Furthermore, the microbial community of sludge tanks were adapted to a wide range of organic compounds, which is explained by the affluent source of nutrients being renewed constantly. To some extent, conditions inside host plants might be similar to this, thus explaining the similarity of the metabolic profiles between both systems. Here, we describe the early events - preceding the establishment of plant-bacterium associations (mainly) from soil - and analyze how bacteria adapt to and colonize niches at the root, how they are transmitted and what the bacterial properties are that improve growth of the host plant.

Recognition/chemotaxis

The sequence of events leading to colonization of a plant by a bacterium that is to become endophytic is presumably similar, at least in the early stages, to that observed for rhizoplane or rhizosphere bacteria. Indeed, bacteria belonging to the so-called 'root-colonizing rhizosphere-competent bacteria' – for example members of the genera *Pseudomonas* (e.g. *P. fluorescens*), *Azospirillum* (e.g. *A. brasilense*) and *Bacillus* (e.g. *B. subtilis*), all common rhizosphere inhabitants - are often found as colonizers of the internal tissue of plants (Hallmann & Berg 2006). Bacterial colonization of roots often starts with the recognition by bacteria of specific compounds that are secreted by the root tissue (Lugtenberg & Dekkers 1999). For instance, tomato roots secreting organic as well as amino acids in their exudates were found to provide chemo-attractants for *P. fluorescens* (strain WCS365), but sugars had no effect on the chemotactic response (de Weert et al. 2002).

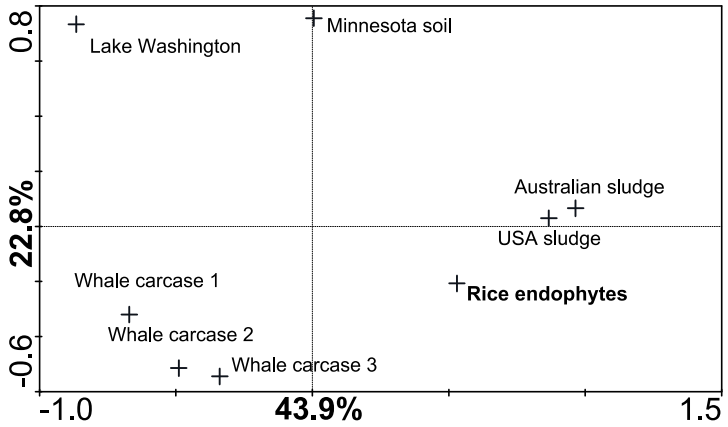


Fig. 1 PCA analysis of COGs profiles of selected metabiomes

The assigned COGs entities from selected metagenome habitats: soil (Tringe et al., 2005), Australian and USA sludge (Garcia Martin et al., 2006), whale carcase (Tringe et al., 2005) and fresh water in lake Washington (Kalyuzhnaya et al., 2008) is shown. The translated protein profile of the rice endophytic community was distinct from other prokaryotic communities.

Bacteria sense, and regulate the response to, their surrounding environment via one- and two-component sensor systems (Faure et al. 2009). One-component systems are typically constituted of single proteins with input and output transmembrane domains, which lack a receiver domain and the phosphotransfer histidine kinase found in two-compound systems. Many one- and two-component systems have been identified to be involved in the recognition of root-exuded compounds, leading to active root colonization. Motility driven by chemotaxis is one of the most important and well understood bacterial systems involved in plant-bacterium interactions. The histidine kinase CheA is responsible for the recognition of chemo-attractants, while the response regulator CheY coordinates bacterial motility via flagellum-mediated chemotaxis (Szurmant & Ordal 2004). Pseudomonads as well as enteric bacteria harbour the GacS/GacA two-component regulatory system, in which GacS, the sensor kinase, recognizes still-unknown environmental signals, and GacA, the transcriptional regulator, activates the production of secondary metabolites and extracellular enzymes that enhance host colonization fitness (Heeb & Haas 2001). Recognition of legume flavonoids by the cytoplasmic membrane-associated NodD protein from rhizobia activates the transcriptional regulator LysR, leading to the production of lipochito-oligosaccharides which induce nodule formation in the host (Brencic & Winans 2005). The Nod factor is probably one of the best-known one-component systems. The one- and two-component sensor/response systems combined with other cross-regulation systems permit bacteria to perform complex information processing, allowing to coordinate appropriate responses in the dynamic rhizosphere environment.

Many biotic and abiotic factors affect root exudation. Spatial and temporal exudation patterns have been observed along the axes of the roots, creating differential niches for diverse soil bacteria (Kuzyakov 2002). Hence, one might hypothesize that different root zones (i.e. the cork zone, root hair, elongation zone, differentiation zone and root cap) create a range of spatial niches that select specific bacterial communities, allowing to establish interactions with the plant. For instance, colonization of wheat roots by *A. brasilense* strain 245 occurs preferentially at the root hair zone and at the sites of lateral root emergence (vande Broek et al. 1993), while colonization of rice roots by *Azoarcus* sp. strain BH72 occurs preferentially in the zones of division and elongation just behind the root cap (Hurek et al. 1994) or - for rhizobial species - at those of lateral root emergence (Chi et al. 2005). Surprisingly, during growth of the root, the root cap cells are sloughed off and, while still alive (i.e. detached living cells known as border cells), they function by attracting and stimulating the growth of beneficial microorganisms, whereas repelling and inhibiting pathogenic ones (Hawes et al. 1998). Moreover, plant traits and physiological states have been shown to affect the composition and diversity of rhizobacterial communities (Hartmann et al. 2009). The effect of bacterial colonization altering root exudates was nicely demonstrated by Rudrappa et al. (2008). The introduction of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 onto leaves of *Arabidopsis thaliana* induced the exudation of malic acid from the roots, which promotes (in a dose-dependent manner) chemotaxis, motility and biofilm formation of *B. subtilis* FB17, thus enhancing root colonization. No biofilm formation of *B. subtilis* FB17 was observed when *Arabidopsis* was inoculated with the non-host bacterium *Pseudomonas syringae* NPS3121, suggesting that the establishment of colonization is specific to a defined bacterial infection regime.

The ability of soil bacteria to approach plant roots via chemotaxis-induced motility and effectively colonize these via attachment and microcolony formation is probably among the strongest deterministic factors for successful endophytic colonization (Compant et al. 2010). In wheat roots, the proportion of isolated bacteria with flagellar motility gradually increased from the rhizosphere to the endosphere (Czaban et al. 2007). A recent metagenomic survey of the endophytic bacterial community obtained from healthy rice root tissues revealed that all compounds of the flagellar apparatus were present in higher abundance than in other metagenomes except for the termite gut microflora metagenome (**Chapter 7**). Furthermore, the importance of chemotaxis-induced motility for root colonization was demonstrated by analyzing *cheA* mutants of *P. fluorescens* WCS365,

which retained motility even thought they were defective in flagellum-driven chemotaxis (de Weert et al. 2002). In the competitive root colonization assay, the *cheA* mutants revealed reduced ability to compete with the wild-type strains. Besides organic and amino acids, plant secondary metabolites, especially flavonoids, have been proposed as important chemoattractants for endophytic colonization. The incorporation of flavonoids in the growth medium enhanced root colonization of rice and wheat by the endophytic bacteria *Serratia* sp. EDA2 and *Azorhizobium caulinodans* ORS571, respectively (Balachandar et al. 2006; Webster et al. 1998). Intercellular root colonization of *A. thaliana* by two diazotrophic bacteria - *A. caulinodans* ORS571 and *Herbaspirillum seropedicae* Z67 - was also stimulated by the application of the flavonoids naringenin and daidzein in low concentrations (Gough et al. 1997). These results suggest that specific classes of flavonoids might be involved in the initial signalling for beneficial plant-bacterium interactions.

Endophytic colonization

In the vicinity of plant roots, competent bacterial endophytes need to gear their metabolisms towards a physiological state that enables optimal nutrient acquisition, niche adaptation and competition. Indeed, several studies on gene expression in rhizobacteria have shown that the genes involved in nutrient acquisition and stress adaptation, next to activation of transcriptional regulators, are among the first responders when bacteria are exposed to root exudate compounds (Somers et al. 2004). Hence, bacterial traits involved in the response to environmental stimuli (e.g. transcriptional regulators), communication (e.g. autoinducers), niche adaptation and plant colonization are important for successful interactions with the plant, in a complex process.

Transcriptional regulators

Bacterial responses to environmental cues must be in perfect synchrony with their metabolic functions and, therefore, transcriptional regulators play important roles in bacterial fitness upon interaction with the plant. The importance of transcriptional regulators in bacteria involved in root colonization was recently demonstrated by English et al. (2010). The authors inserted a transposon upstream of the *hns* gene from *Enterobacter cloacae* UW5, which increased gene expression when the strain was exposed to canola roots. Although the levels of *hns* transcripts were only up to twofold higher, the mutant strain increased its root colonization and even outcompeted the wild-type strain in a direct competition assay. The *hns* gene encodes the small histone-like protein H-NS that

binds predominantly to AT-rich sequences of DNA, regions that are commonly found in promoter sequences (English et al. 2010). Adaptation to environmental stimuli occurred within minutes in *Salmonella enterica* subsp. *enterica,* where several H-NS-dependent genes were upregulated with the increase of temperature including the flagellar/chemotaxis regulon (Ono et al. 2005).

The rice endophyte microbiome comprises a high diversity and a high abundance of transcriptional regulators, which is only exceeded by the human gut metagenome (**Chapter 7**). A subset of three transcriptional regulators (i.e. belonging to the LysR-, Crp- and IclR-families) was strongly overrepresented in the rice metagenome. The physiological responses affected by these transcriptional regulators are broad, comprising the metabolism of sugars and amino acids, transport processes, virulence, quorum sensing, pilus synthesis and motility (Korner et al. 2003; Maddocks & Oyston 2008; Molina-Henares et al. 2006). This suggests a very high degree of plasticity of responses to varying environmental stimuli, such as those represented by plant compounds.

Adaptation to the niche and adhesion

Given the fact that plant-derived compounds are the main N- and C-sources for heterotrophic soil bacteria, rhizosphere/rhizoplane bacteria (rhizobacteria), to be successful, must rapidly adapt their metabolism to the range of available nutrients. Gene expression analyses of the root-colonizing bacterium *Pseudomonas putida* KT2440 have revealed an upregulation of genes involved in metabolism and stress adaptation in the rhizosphere of corn plants (Matilla et al. 2007). Specifically, genes involved in the uptake of 'readily-available' root exudate compounds (e.g. amino acids, dipeptides and polyamines), and aromatic compounds (e.g. phenylacetic and/or phenylalkanoic acids, plant exopolymers β-glucosidase and urease), as well as those encoding responses to stress (e.g. glutathione peroxidase and fatty acid *cis-trans* isomerase) and detoxification of proteins (e.g. putative efflux transporters) were upregulated. Corroborating these results, the analysis of the rice endophyte metagenome revealed a high abundance of genes involved in transport systems, mainly ATP-binding cassette (ABC) family transporters for several amino acids or polyamines as well as genes involved in the degradation of aliphatic and aromatic compounds, when compared with other selected metagenomes (**Chapter 7**). Furthermore, the rice endophyte metagenome contained an extremely high number and diversity of genes encoding enzymes potentially involved in the detoxification of reactive oxygen species (ROS), glutathione synthases and glutathione-S-transferases (GST)

(**Chapter 7**).These results suggest that bacterial endophytes might be selected by harbouring a wide range of metabolic pathways, in which by taking up the secreted metabolite waste, they might ameliorate plant stress.

Adaptation to oxic versus anoxic conditions is often required for a bacterium to survive in the vicinity of roots, especially at plants growing in flooded ecosystems. Under flooded conditions, plants like rice form heterogeneous oxic/anoxic interfaces which might create opportunities for rhizobacteria and endophytes able to perform fermentation processes (Brune et al. 2000). Under anoxic conditions, rice is known to accumulate ethanol, lactic acid and alanine at root tissues. Ethanol is one of the major carbon sources for the endophytic bacterium *Azoarcus* sp. strain BH72, whose genome harbours ten genes encoding putative alcohol dehydrogenases (Krause et al. 2006). The secretion of phytotoxic levels of ethanol may have created a niche opportunity for *Azoarcus* to colonize rice roots. This observation corroborates the data from the rice endophyte metagenome analysis, where genes involved in fermentative abilities were overrepresented (**Chapter 7**).

Upon root surface, the bacterial adhesion is mediated by cell surface structures such as polysaccharides, pili and adhesins (Hori & Matsumoto 2010). Genome analysis of *Enterobacter* sp. strain 638, a competent bacterial endophyte of poplar, revealed the presence of many genes encoding putative proteins involved in root adhesion, including hemagglutinins, curly fibers, autotransporter adhesin (YadA), type I and IV pili, cellulose biosynthesis and capsular polysaccharides (Taghavi et al. 2010). Interestingly, a number of these genes are present in genomic islands or on plasmids, suggesting their acquisition by horizontal gene transfer. The diazotrophic *A. brasilense* strain Cd has a major outer membrane protein (MOMP) involved in early host recognition (Burdman et al. 2001). MOMPs from *A. brasilense* Cd strongly adhere to root extracts of cereal plants when compared to legumes. The authors speculated that MOMPs may act as adhesins and therefore are involved in adsorption and cell aggregation on roots of selected host. Another cell surface structure, the type IV pilus, is also involved in the establishment of the endophytic bacterium *Azoarcus* sp. BH72 on the surface of rice seedling roots (Dorr et al. 1998). The mutant strains *pilA* and *pilB* (defective in pilus formation) were impaired in their proper adherence and colonization of rice roots. The role of bacterial cell surface polysaccharides [e.g. lipopolysaccharides (LPS), exopolysaccharides (EPS), capsule and peptidoglycan] in plant colonization is currently unknown. However, many of those genes were identified in the genomes of endophytic bacteria (Krause et al. 2006; Fouts et al. 2008; Bertalan et al. 2009; Taghavi et al. 2010). It is interesting that the endophytic

bacterium *Azoarcus* sp. BH72 harbours many genes involved in the synthesis of cell surface compounds but there is no gene encoding this activity in the genome of the closely-related soil isolate *Azoarcus* sp. EbN1, suggesting the role of cell surface polysaccharides in the invasion/interaction of endophytes with the plant host.

Once on the root surface, bacteria might use a different type of motility, known as twitching motility, to reach their favourite entry sites (e.g. sites of lateral root emergence, root tips and/or pathogen- or predation-induced wounds). Twitching motility is mediated by type IV pili, which extend from the poles of a bacterial cell and retract, pulling forward the cell. Endophytic colonization of rice roots by the diazotrophic *Azoarcus* sp. BH72 was completely impaired in a *pilT* mutant, defective for pilus retraction, although partial colonization (50%) was observed on the root surface (Bohm et al. 2007).

Endorhizal colonization

Before entering the plant internal tissues, soil bacteria colonize the rhizodermal cells. The colonization strategy varies for each bacterium - host interaction. A recent histochemical study with three bacterial species colonizing the roots of sugar beet revealed that each strain has a distinct colonization pattern (Zachow et al. 2010). For instance, *P. fluorescens* L13-6-12 and *P. trivialis* RE1-1-14 formed microcolonies (i.e. tens to hundreds bacterial cells), respectively, on the upper parts of the roots and in compartments between root cells, as well as upon emergence of lateral roots, whereas *Serratia plymuthica* 3Re4-18 colonized - as single cells - the entire root surface as well as internal root tissues. The authors showed that each bacterial species occupied specific niches and morphologically detectable interactions were rare. These results suggest that each bacterium has its own preferred colonization sites, which may overlap in field conditions. Hence, stacking of various facilitating bacterial traits might be important for successful colonization.

Communication via quorum-sensing (QS) is one of the most important bacterial traits to coordinate population behaviour (von Bodman et al. 2003). Bacterial communication by autoinducer molecules plays an essential role in endophytic colonization. QS mutant strains of *B. kururiensis* M130, impaired to produce and respond to one type of *N*-acyl homoserine lactone (AHL), showed decreased root and aerial rice tissue colonization when compared to the wild-type (Suarez-Moreno et al. 2010). Furthermore, the beneficial effects of endophytic colonization (i.e. increases in root length and branching) were reduced in QS mutant strains. Bacterial signal molecules such as lipochito-oligosaccharides and lumichrome are potentially involved in host growth stimulation (reviewed in Mehboob et

al. 2009). By using the quorum quenching approach, Boyer et al. (2008) showed that a mutant of the rice endophyte *Azospirillum lipoferum* B518 that constitutively expressed AttM lactonase (an enzyme that hydrolyzes the lactone ring of AHLs) increased the synthesis of proteins linked to transport and chemotaxis. This suggests that QS in this strain is dedicated to regulate functions involved in root colonization. In the aforementioned rice endophyte metagenome survey, genes encoding proteins for autoinducer synthesis and detection were highly abundant, with three different autoinducer systems being identified [i.e. autoinducer-2 system (AI-2), the diffusible signal factor system (DSF) and the AHL system]. This probably reflects a need for concerted gene regulation for virulence and colonization by endophytic bacteria (**Chapter 7**).

Many bacterial pathogens and symbionts might secrete or inject proteins (called effectors) to interact with plant cells. The function of effectors secreted by symbionts is still unknown but they often differ from those from pathogens (Deakin and Broughton 2009). In the rice endophyte metagenome, all known protein secretion systems for translocation across the cytoplasmic and outer membranes were present except for compounds of the type III secretion system (T3SS; **Chapter 7**). Striking was the high abundance of genes encoding compounds of type VI secretion systems (T6SS). T6SS is involved in a broad variety of functions, from eukaryotic host infection to biofilm formation and response to stress (Bernard et al. 2010) and might be important for the endophytic lifestyle.

Soil bacteria can enter the epidermal root tissues by two processes: passively, for instance by penetrating sites at the junction of adjacent epidermal cells (Benhamou et al. 1996) and sites at the emergence of lateral roots (Govindarajan et al. 2008) or actively, with the production of hydrolytic enzymes (e.g. exoglucanase, endoglucanase and endopolygalacturonase) involved in plant cell wall degradation (Reinhold-Hurek et al. 1993; Compant et al. 2005b). It has been proposed that the levels of cell-wall-degrading enzymes produced by root-colonizing bacteria differentiate endophytes (low levels) from phytopathogens (deleteriously high levels) (Elbeltagy et al. 2000). Although this assumption has not been proven, it makes sense if the invader microorganisms need to avoid triggering the plant defence system. Genes encoding plant polymer-degrading enzymes were observed in high abundance and diversity in the rice endophyte metagenome (**Chapter 7**). They may contribute to endophyte entry into and spread inside the plant tissue.

Systemic colonization

A subset of endophytic bacteria is able to colonize the aerial parts of its host plant from the root tissue (**Chapter 2**, Hardoim et al., 2008, Compant et al., 2010) and even systemically colonize stem and leaf tissues. Bacterial densities in stem and leaf tissues are considerable lower than in roots, typically 10^3-10^4 colony-forming unity (CFU) g^{-1} tissue. Moreover, the endobacterial diversity is also lower, indicating the need for highly specialized adaptive traits that allow thriving in the photosynthetic tissues (Hallman, 2001). Furthermore, the endobacterial populations inhabiting the aerial parts are mostly derived from the endorhiza via systemic spread via xylem vessels or via intercellular spaces of parencymatic tissue. However, as with phytopathogenic bacteria, entry from the phyllosphere via stomata or hydathodes can also occur; this has received very little attention thus far.

Vegetative transmission

In addition to invasion of root/shoot tissue, bacteria can also be introduced into plants via propagated vegetative material (e.g. seeds, cuttings, stems, tissue culture) and thus spread to descendent generations (Hallmann et al. 1997). Although bacteria can be absent or present in very low densities in reproductive organs (10^1-10^3 CFU/g tissue; Nissinen et al., unpublished; Compant et al., 2010), seeds from many plant hosts are seen as important vectors for endophytic dissemination (Mundt and Hinkle 1976). Vertical transmission of endophytes has been observed by isolation of bacteria from cotton and rice seedlings growing aseptically on agar medium (Adams and Kloepper 2002; **Chapter 6**). Furthermore, the isolation of bacteria from surface tissue and surrounding medium of rice seedlings growing aseptically on agar medium (Kaga et al. 2009) suggested that, once seeds are germinated, bacterial endophytes may move out and even colonize the surrounding plant sites. Thus, one might speculate that seed transmission of selected endophytes may be needed for plant establishment in hostile soil. This assumption was strengthened by the isolation of bacterial endophytes that were transmitted via seeds, which subsequently were found to assist the cactus seedlings to establish and grow on barren rock (Puente et al. 2009a). The dissemination of endophytic bacteria via seeds might thus be more common than previously considered, and might even pose an ecological advantage for the host as plants carrying the beneficial bacteria can thus foray adverse conditions (Lopez-Lopez et al. 2010). Further studies are needed to confirm these exciting leads and assumptions.

Plant beneficial properties

Plant growth promoting (PGP) properties of rhizosphere bacteria have been intensively studied and are well documented. However, the agricultural applications of PGP rhizobacteria have often led to less than optimal results. This might be due to a recurrent inability of added plant gowth-promoting bacteria (PGPB) to thrive and compete with the native soil microbiota and successfully colonize the rhizosphere (Garbeva et al. 2004). These findings, combined with the recent discovery of the high diversity and abundance of endophytic bacteria has tremendously increased the interest in the PGP potential of endophytic bacteria. Bacterial endophytes have been shown to enhance plant growth by improving the mobilization and uptake of nutrients, by increasing stress tolerance and growth via production or (co)regulation of phytohormones and by enhancing plant disease resistance by antagonism, competition or by inducing or priming the plant's own defence systems (Compant et al. 2010).

Nutrient status

Plants acts as "miners" of Earth's crust/soils, acquiring essential nutrients for their growth mainly through root systems. Among the essential nutrients, nitrogen and phosphorus are needed in relatively high quantities, however the availability of these elements is often limited in soil. Bacterial endophytes might help their host plants to acquire these nutrients.

Nitrogen

Nitrogen-fixing (diazotrophic) symbionts, i.e. nodule-forming rhizobia and actinobacteria, are well known and often represent highly significant N input in their respective plant hosts, in particular in nitrogen-poor soils. Additionally, diazotrophic bacteria have been isolated from numerous gramineous host plants, suggesting that they actively participate in biological N_2 fixation. Significant amounts of N have been shown to be incorporated into key agronomical crops like rice, sugarcane and maize by biological N_2 fixation. Although studies have shown in vivo expression in diazotrophs of the genes encoding nitrogenase and incorporation of $^{15}N_2$ gas into the host, it is still questionable whether the incorporated N is mainly due to the death and mineralization of diazotrophs or through direct and rapid transfer, as occurs in legume nodules (James 2000). Nevertheless, selected diazotrophic bacteria such as *Burkholderia* spp., *Azoarcus* sp. BH72, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense* have been reported to

significantly increase the host biomass production under controlled conditions by N_2 fixation (Bhattacharjee et al. 2008).

Studies on the diversity and community composition of associative N-fixing bacteria are common and practically every phylum contains species harbouring nitrogenase. Furthermore, this enzyme is conserved through evolution with ample evidence of lateral gene transfer. Thus, as only a small fraction of soil bacteria colonize gramineous plants, particular associative diazotrophic bacteria might be considered as true and successful symbionts. Endophytic diazotrophic bacteria, particularly *Gluconacetobacter diazotrophicus*, *Burkholderia spp* and *Herbaspirillum seropedicae*, have been extensively found, e.g. in Brazilian sugarcane (*Saccharum* spp.) cultivars (Baldani and Baldani 2005). Similarly, *Azoarcus* sp. BH72 might be responsible for N_2 fixation in Kallar grass and rice (Hurek and Reinhold-Hurek 2003).

Phosphate

Phosphorus is one of the major plant-growth-limiting nutrients. It is likely to become more important, as the available sources of phosphorus on Earth are getting sparse. Phosphates applied to agricultural soils are rapidly immobilized and rendered inaccessible for plants. Due to this rapid immobilization, many agricultural soils have large reservoirs of phosphates, however in an inaccessible form (Rodriguez and Fraga 1999). Many PGPB can solubilize inorganic phosphates by secretion of organic acids, making them accessible to host plant. Phosphate solubilization is a common trait among plant-endophytic bacteria. For instance, the majority of endophytic populations from strawberry, soybean and other legumes, sunflower and cactus (59-100%) were able to solubilize mineral phosphates in plate assays (Dias et al. 2009; Kuklinsky-Sobral et al. 2004; Palaniappan et al. 2010; Forchetti et al. 2007; Puente et al. 2009b).

A survey of bacterial endophytes from sunflowers grown in irrigated or drought regime revealed that more phosphate-solubilizing endophytic bacteria were isolated from drought-exposed plants, suggesting selection for such PGPB in stress conditions (Forchetti et al. 2007). All phosphate-solubilizing bacteria (PSB) also revealed other plant-beneficial properties, including the ability to grow on nitrogen-free medium and the production of several phytohormones. In addition to environmental pressure, phosphate-solubilizing endophytes might be favourable in the active growth stages of plants. Kuklinsky-Sobral et al. (2004) analyzed epi- and endo-phytic isolates from several growth stages and cultivars of soybean. They found that 60% of the endobacterial isolates (representing dominantly

Pseudomonaceae, *Burkholderiaceae* and *Enterobacteriaceae*) from the early plant growth stages were phosphate solubilizers, compared to less than 50% of the isolates from senescent plants. The majority of the phosphate-mobilizing isolates were also able to fix nitrogen and produce indole-3-acetic acid (IAA). Likewise, Palaniappan et al. (2010) isolated endophytic bacteria from root nodules of the fabaceous plant species *Lespedeza* and found that the majority of endophytes were able to solubilize phosphates. The authors also found that most of the endobacterial isolates harboured multiple PGP properties (i.e. phosphate solubilization, IAA and siderophore production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity).

Some controversy has surfaced about whether phosphate solubilization per se is plant beneficial, as most PGP endophytes have multiple PGP properties, as highlighted above. However, a clear correlation between phosphate mobilization and plant growth has been shown in several studies. Dias et al. (2009) analyzed endobacterial isolates from strawberry, mostly representing *Bacillus subtilis* and *B. megaterium*, that were all able to solubilize calcium phosphate in plate assays. The phosphate solubilization efficiency varied markedly between isolates. The plant growth promotion capacity of the isolates correlated with their phosphate solubilization activity, as well as with IAA production. Puente et al. (2009b) isolated and analyzed endophytic bacteria from cardon cactus, a pioneer desert plant able to establish on solid rock. The majority of endophytes was capable of solubilizing Fe/Ca -phosphates and pulverizing rock. As these bacteria were also present in cactus seeds, from where they colonized the rhizosphere of the developing seedlings, they might have a role in desert colonization and soil formation. It should be noted that many of the phosphate solubilizing isolates were also diazotrophic, thus providing the host plant with N next to P (Puente et al. 2009a). The endophytes were tested in pot experiments, where endophyte-free cacti growing on mineral phosphate rock were amended with endophytes or nutrients, or were grown under sterile conditions. The bacterized plants grew well without nutrient addition and were comparable to fertilized plants, whereas the endophyte-free cacti failed to develop. This indicated that the endophytes were able to provide the developing plantlets with phosphate as well as nitrogen (Puente et al., 2009b).

Other nutrients

Albeit less well studied, iron chelation (via siderophores) is a common trait in endophytic bacterial communities. For instance, the rice endophyte metagenome revealed a high

number of genes encoding proteins potentially involved in siderophore biosynthesis, ferric-siderophore membrane receptors, iron uptake transporters and storage proteins (**Chapter 7**). As iron is fiercely competed for in soil as well as within eukaryotic host tissues, iron-chelating bacteria can deprive putative pathogens of available iron, therefore exerting antagonistic activity.

As bacteria mobilize mineral phosphates by secretion of organic acids, they are likely also able to mobilize other mineral nutrients. *Gluconacetobacter diazotrophicus* PAl5 is a PGPB with many PGP properties and it is able to solubilize zinc from zinc oxides and phosphates, in addition to calcium phosphates (Saravanan et al. 2007). The zinc mobilization activity is dependent on carbon availability for *G. diazotrophicus* PAl5. However, mobilization of other mineral nutrients than phosphorus by endophytes has been very little screened for.

Plant growth enhancement

In addition to improving the plant nutrient status, endophytic bacteria might stimulate plant growth by directly producing phytohormones, other growth regulators (e.g. lipochito-oligosaccharides and lumichrome; reviewed in Mehboob et al. 2009) and enhancing host anabolism (e.g. photosynthesis ability), or by regulating plant phytohormone levels (Fig. 2).

Production of IAA and other hormones

Auxins, of which IAA is most common, are phytohormones necessary for plant growth and morphological development, including cell elongation, maintenance of apical dominance, formation of vascular tissues, cell elongation and prevention of senescence. Auxins also counteract root apical dominance by cytokinins (CKs) and promote the formation of lateral roots and the root system. Further, IAA prevents the formation of ethylene (ET) in low concentrations, but stimulates ET synthesis in high concentrations (Woodward and Bartel 2005).

IAA production is a common trait among endophytic bacteria, and IAA producing endophytes representing a vast range of bacterial phyla/classes have been isolated from multiple plants, including poplar, soybean, epiphytic and terrestrial orchids, cactus, potato and strawberry. IAA production by endophytic bacteria has been associated with the promotion of plant root growth, enhanced production of lateral roots and increases in root volume and biomass (Taghavi et al. 2009, Kuklinsky-Sobral et al. 2004, Tsavkelova et al.

2007, Dias et al. 2009). IAA producing bacteria are commonly isolated from both the rhizo- as well as the endosphere. Tsavkelova et al. (2007) isolated and analyzed endophytic and rhizoplane bacteria from epiphytic as well as terrestrial orchids. The endobacterial isolates, representing the genera *Erwinia*, *Bacillus*, *Pseudomonas* and *Flavobacterium*, all produced IAA. Further, on average, the endobacterial communities yielded more efficient IAA producers (as measured in cultures) than rhizoplane ones. When tested on kidney beans, supernatants from endophyte cultures significantly stimulated root formation and resulted in increases in root length as well as the number of developing roots, indicating the potential role of endobacterial auxins in root development (Tsavkelova et al. 2007).

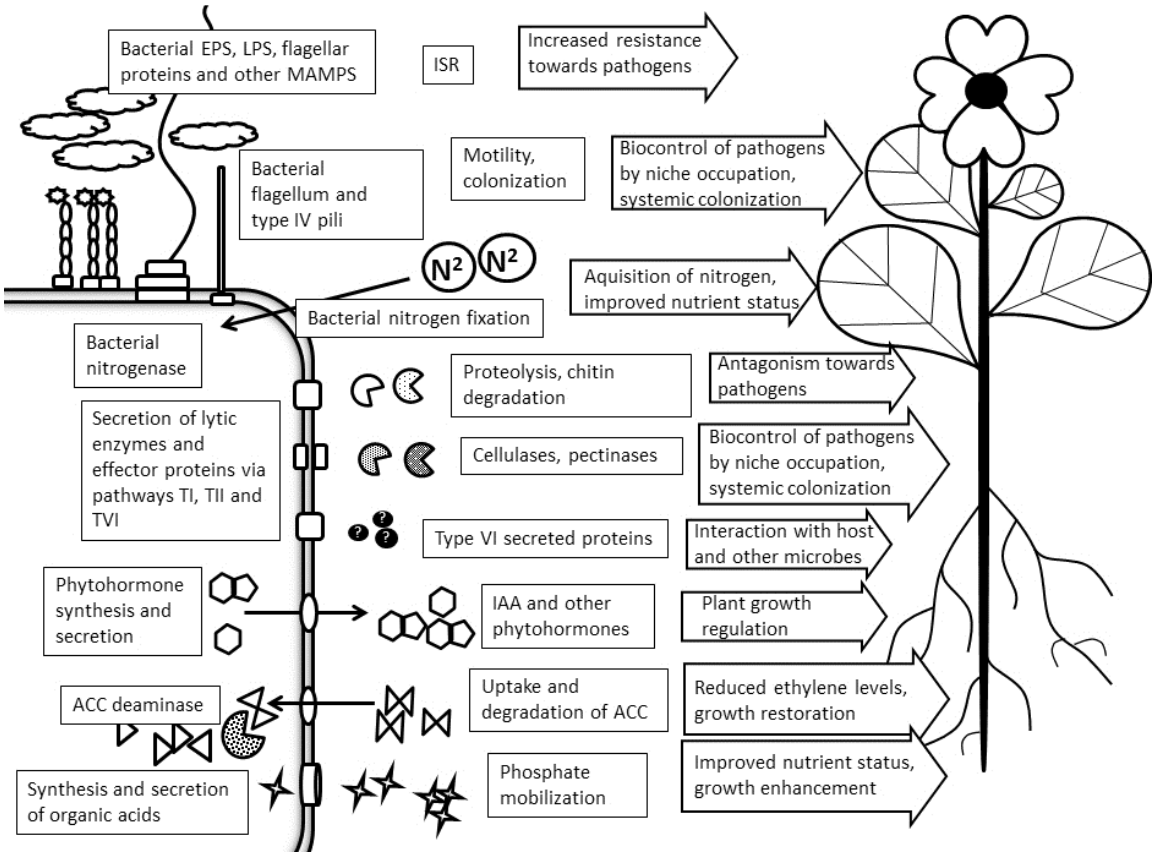


Fig. 2 Endophytic bacterial properties and their plant beneficial effects (block arrows), based on current knowledge. EPS= extracellular polysaccharide; LPS=lipopolysaccharides; MAMP= microbe associates molecular pattern; IRS= induced systemic resistance; TI= type one protein secretion system; TII= type two protein secretion system; TVI= type six protein secretion system; IAA= indole-3-acetic acid; ACC= 1-aminocyclopropane-1-carboxylate

The production of IAA in PGPB is often associated with other beneficial properties. Thus, the role of IAA production has rarely been directly proven. The promotion of root growth and lateral root formation by plant beneficial *Pseudomonas putida* GR12-2 was shown to be dependent on the presence of a functional IAA biosynthesis pathway, as plant

growth promotion potential was lost in a *P. putida* GR12-2 IAA synthesis mutant (Patten and Glick 2002).

Enhancement of photosynthetic activity

Bacterial endophytes can actively alter the physiology of the host plant. Introduction of different rhizobial species, *A. caulinodans* ORS 571, *Sinorhizobium meliloti* 1021 and *Mesorhizobium huakui* 93 enhanced rice growth by stimulating photosynthetic activity and enhancing resistance to drought (Chi et al. 2005). Further studies revealed that *S. meliloti* 1021 induced the production of photosynthesis-related proteins in rice plants. Using a proteomic approach, Chi et al. (2010) showed that proteins related to Rubisco activase, pyruvate orthophosphate dikinase (catalyses the production of PEP and involved in the light and dark reactions), transport of nuclear-encoded proteins and nutrients to the chloroplast, were upregulated in the presence of the endophyte.

The promotion of photosynthetic capacity is not limited to rice/rhizobia associations. Introduction of three endophytic bacteria, i.e. *Bacillus pumilus* 2-1, *Chryseobacterium indologene* 2-2, and *Acinetobacter johnsonii* 3-1, in sugar beet increased the plant chlorophyll content, leading to an enhanced carbohydrate synthesis when compared with uninoculated plants (Shi et al. 2010). The authors speculated that the production of unidentified compounds by the endophytes might have led to an enhancement of electron transport and, consequently, promotion of chloroplast metabolism.

Regulation of ethylene levels by ACC deaminase-producing bacteria

ET is a highly versatile plant hormone, which is involved, for example, in seed germination, fruit ripening, formation of mature xylem vessels and root hairs as well as in senescence of flowers and leaves. In plants, ET is synthesized from methionine via a two-step pathway. The immediate precursor is a non-protein amino acid ACC, which is oxidized to ET. Synthesis of ACC and ET is induced by several abiotic and biotic stressors, including flooding or drought, pathogen attack or wounding. Additionally, ET synthesis is induced by auxins, especially IAA and by CKs and inhibited by abscisic acid (ABA). ET has pleiotropic effects, and the response to ET is dependent on the type of plant tissue, its growth state and physiological environment. However, excessive production of ET associated with the stress response also inhibits root elongation and growth. Additionally, ET has been shown to direct auxin transport and signalling (Strader et al. 2010).

A group of plant-associated bacteria is able to degrade the ET precursor ACC by (bacterially-encoded) ACC deaminase and utilize the end products as carbon and nitrogen sources. Hence, this forms an efficient sink for ACC. Concomitantly, these bacteria lower the ET levels in colonized plant tissue and restore plant growth under stressful conditions (reviewed in Glick et al. 2007). Production of ACC deaminase and associated plant growth promotion by root elongation and increase of plant biomass has been reported for numerous endophytic species, including many *Burkholderia phytofirmans* and *B. cepacia* isolates, *Methylobacterium fujisawaense,* as well as for *Pseudomonas, Arthrobacter* and *Bacillus* spp. (refer to Nadeem et al. 2010 for extensive list of PGPB with ACC deaminase activity)

Abiotic stress - Environmental stressors, such as soil salinity, extremely high or low temperature, freezing, drought, flooding or anoxia, often inhibit plant growth either directly by interfering with normal plant functioning or indirectly by the synthesis of excess stress-related ET and subsequent growth inhibition. Soil salinity is the major abiotic stressor in plants, being around 20% of the world's cultivated lands salt-affected. High concentrations of salts cause ion imbalances leading to hyperosmotic stress in plants. Another stressor, low temperature (i.e. just above freezing) causes chilling stress in many tropical or sub-tropical plants. Chilling injuries including retarded growth, leaf lesions and wilting and goes with loss of cell membrane properties ensuing from changes in membrane fluidity. Salt and cold stresses are closely related to other abiotic stresses and associated with e.g. elevated ET levels and stunted growth.

Numerous studies link the beneficial effects of inoculation with ACC deaminase producing endophytic bacteria with increased stress tolerance and growth in suboptimal conditions. The inoculation of tomato, cotton, groundnut, canola, maize and wheat with the ACC-deaminase producing bacteria *Achromobacter piechaudii* AVR8, *Klebsiella oxytoca* Rs-5, *Serratia proteamaculans* M35, *Enterobacter cloacae* CAL2 and *Pseudomonas* spp. increased host biomass production, lowered Na^+ and enhanced K^+ cell content compared to uninoculated plants (reviewed in Nadeem et al. 2010). *Burkholderia phytofirmans* PsJN is an intensively studied endophyte that has been associated with growth promotion and enhanced stress tolerance in several plant species, including potato, vegetables and grapevine (Sessitsch et al. 2005). *B. phytofirmans* PsJN has ACC deaminase activity and the plant growth enhancement under environmental stress has been postulated to be associated with ACC deaminase production by the bacterium. PsJN-inoculated grapevines showed a 10-fold increase in root growth at both 26 and 4 ºC. The enhanced growth was

associated with an increase in plant photosynthetic capacity and starch content, as well as proline and phenolic contents in plant cells. This indicated enhanced cold tolerance of plants by PsJN inoculation (Ait Barka et al. 2006). An *acdS* mutant of *B. phytofirmans* PsJN, which was deficient in ACC deaminase activity, also lost its ability to promote root elongation in canola seedlings. Curiously, this ACC deaminase mutant also synthesized a decreased level of siderophores and increased amounts of IAA, which were suggested to result from increased levels of stationary phase sigma factor RpoS (Sun et al. 2009). This indicated co-regulation of ACC deaminase synthesis, siderophore and IAA production. Complementation with functional *acdS* restored both the ACC deaminase production and root elongation capacity, offering direct proof of the role of bacterial ACC degradation in plant growth enhancement. Curiously, complementation (in trans) did not reverse the IAA and siderophore phenotypes (Sun et al. 2009).

Inoculation of the alpine plant species *Chorispora bungeaana* with endophytic *Clavibacter* sp. Enf12 isolated from the same plant growing under snow enhanced plant growth both at 20 and 0 ºC. It also significantly attenuated the production of ROS, oxidative damage and electrolyte leakage. Inoculation also led to elevated levels of antioxidant enzymes and proline, indicating improved control of oxidative damage and increased hardiness (Ding et al. 2011). Similarly, a cold-tolerant *Serratia marcescens* SRM isolate from summer squash significantly enhanced biomass and nutrient uptake in wheat seedlings under cold conditions. *S. marcescens* has several PGP capacities, including IAA production and phosphate solubilization, and these activities are retained at 4 ºC (Selvakumar et al. 2008).

Moreover, in addition to ACC deaminase and ET levels, other endobacterial factors are likely to play roles in plant stress tolerance and growth. Sziderics et al. (2007) studied the effect of five ACC deaminase producing endophytes on the adaptation to abiotic stress by pepper (*Capsicum annuum*). Under moderate stress, four of the five isolates increased plant biomass. *Microbacterium* sp. EZB22, the only studied strain devoid of IAA production, failed to promote growth, despite its ACC deaminase activity, indicating that growth enhancement is likely due to several bacterial PGPs. *Bacillus* sp. EZB8 and *Arthrobacter* sp. EZB4 were able to attenuate the induction of several stress-related genes in pepper, indicating reduced stress (Sziderics et al. 2007).

Resistance to heavy metals and other toxic compounds

A new field has recently emerged, focusing on the role of the plant-endophyte partnership in the remediation of heavy metal contaminated soils. Endophytes offer several advantages to rhizobacteria: they are better maintained (less competition), they occur in a pollution gradient (plant accumulation, harvest possible), and they offer a more specific relationship with host. Therefore, heavy metal resistant bacterial endophytes might have the ability to accumulate and/or sequester heavy metals. Furthermore, such endophytes with appropriate degradation pathways and metabolic capabilities might improve the degradation of organic contaminants and reduce phytotoxicity. Lastly, stress-ameliorating endophytes might assist their hosts to overcome contaminant-induced stress responses and PGP endophytes might improve plant growth and thus contaminant extraction from soil or water (Weyens et al. 2009a).

Heavy metals

The assessment of the culturable bacterial community from the Ni hyperaccumulator *Thlaspi goesingense* revealed that the endophytic community tolerated high levels of Ni and many endophytic strains were able to grow on ACC as sole N source when compared to those isolated from the rhizosphere (Idris et al. 2004). Sun et al. (2010) revealed that the beneficial effect of endophytic bacteria from one host plant species could be applied to another plant species. The Cu-resistant strains *Bacillus megaterium* JL35 (isolated from *Elsholtzia splendens), Sphingomonas* sp. YM22 and *Herbaspirillum* sp. YM23 (both isolated from *Commelina communis)* increased the root dry weight by 132 to 155 % and the aboveground tissue Cu content by 63% to 125% when introduced onto rape (*Brassica napus*) growing in Cu-contaminated substrate. Many endophytic bacteria that are resistant to one metal show resistance to other metals as well (Kabagale et al. 2010). Hence, they might be used to improve phytoextraction in sites contaminated with multiple metals.

Organic pollutants

Bacteria have two major assets that makes them suitable to combine them with plants in cases of organic pollutant removal: i) heterotrophic bacteria rely on organic compounds as carbon sources and hence they often show a great diversity of metabolic pathways to attain their nutrition, ii) the terminal products of their organic compound metabolism are often CO_2, H_2O and cellular biomass. On the other hand, metabolism of organic compounds by plants consists of a general transformation of more soluble forms and sequestration (Weyens et al. 2009b). The inoculation of poplar with endophytic *Burkholderia cepacia*

VM1468 containing the plasmid pTOM-Bu61, coding for constitutively-expressed toluene degradation, revealed positive effects on plant growth in the presence of toluene and reduced the amount of toluene released via evapotranspiration when compared with poplar inoculated with the soil bacterium *B. cepacia* Bu61 (pTOM-Bu61) or uninoculated plants (Taghavi et al. 2005). Similar results were observed in lupine inoculated with *B. cepacia* L.S.2.4 (pTOM-Bu61), a natural endophyte of yellow lupine (Barac et al. 2004). These results suggest that engineering of endophytic bacteria can be a promising technique to improve phytoremediation of soils contaminated with organic pollutants. Furthermore, pea (*Pisum sativum*) plants inoculated with the endophyte *P. putida* VM1450, a bacterium possessing the metabolic pathway to degrade 2,4-dichlorophenoxyacetic acid (2,4-D), showed higher capacity for 2,4-D removal from soil than uninoculated plants (Germaine et al. 2006). It is interesting that 2,4-D is a selective systemic herbicide for the control of broad-leaved weeds and that pea inoculated with *P. putida* VM1450 showed no 2,4-D accumulation in the aerial tissues. This suggested that the bacterium might help its host by the rapid uptake and degradation of the hazardous compound.

Disease resistance

Endophytic bacteria can protect their host plants from harmful microbes and pests directly by antagonism or competition for the niche (i.e. space and nutrients), or indirectly by upregulating or inducing/priming the plant defense system to respond faster and more efficiently towards invading pathogens.

Antagonism against fungi, nematodes and phytopathogenic bacteria

Direct antagonism towards pathogens can be attained by the production of antifungal substances or fungal growth inhibitors, by antibiotics or other antibacterial metabolites. A wide variety of endophytic bacteria with antagonistic activity against fungal, bacterial and oomycete pathogens have been reported (reviewed in Lodewyckx et al. 2002). *Pseudomonas*, *Bacillus* and *Paenibacillus* spp. and strains of actinobacteria are the most commonly reported species studied as antagonistic against fungal or oomycete pathogens. Some have been successfully tested with respect to disease suppression in a wide diversity of plants, e.g. wheat, potato, black pepper and ginseng (Coombs et al. 2004; Sessitsch et al. 2004; Berg et al. 2005; Aravind et al. 2009; Cho et al. 2007a).

Actinobacteria are known for their production of a wide array of secondary metabolites. Coombs et al. (2004) screened 38 actinobacterial strains isolated from wheat,

representing *Streptomyces*, *Microbispora*, *Micromonospora* and *Nocardioides,* for their antifungal potential against *Rhizoctonia solani*, *Pythium* sp. and *Gaeumannomyces graminis* var *tritici* (the causal agent of take-all disease in wheat) both in vitro and by bioassays. The analyses revealed that 64% of the strains had antifungal properties in in vitro assays, and 17 strains were efficient in planta (in steamed soil) against take-all disease. The active isolates were also effective under field conditions in the biocontrol against take-all as well as *Rhizoctonia* (Coombs et al. 2004).

In contrast to the high proportion of antifungal isolates in actinobacterial endophytes, Sessitsch et al (2004) found that only 0-11% of potato endobacteria possessed activity against three fungal pathogens and the oomycete *Phytophthora cactorum*. The majority of the isolates, however, were effective antagonists against *Streptomyces scabies* and other bacterial pathogens. It is likely that potato scab affecting the potatoes in this study selected for the antagonistic endobacteria. The isolates showing antagonism against fungal as well as bacterial pathogens were from the genera *Pseudomonas*, *Paenibacillus* and *Clavibacter* (an actinobacterium).

Screening of endophytic bacteria from black pepper against *Phytophthora capsici* with three independent methods identified 14-16 antagonistic isolates based on mycelial growth inhibition on agar plate assays, lesion inhibition in cut shoot assay and foot rot suppression in microcosm assays. Three isolates with the best *Phytophthora* antagonistic capacity achieved over 70% disease suppression in greenhouse trials. *P. aeruginosa*, *P. putida* and *Bacillus megaterium* were identified as effective antagonistic endophytes for the control of *Phytophthora* foot rot in black pepper. However, although the disease suppression ability was clearly dependent on the antagonistic capacity against the causal oomycete, disease suppression rates were also dependent on the pepper cultivar (Aravind et al. 2009).

Cho et al. (2007a) analyzed the antifungal activity of 63 endophyte isolates from ginseng against *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythium ultimum* and *P. capsici*. About 50% of the isolates were antagonistic against 2-4 pathogens, and three isolates (*Bacillus* sp., *Paenibacillus polymyxa* and *Pseudomonas poae*) had broad-spectrum antifungal activity and were antagonistic against all tested pathogens.

Induced defences and priming

Plants have a set of nonspecific defence mechanisms to protect them against bacterial, viral and fungal pathogens. Systemic acquired resistance (SAR) is induced by (local) exposure to pathogens. Once induced, SAR is active against a broad range of pathogens. SAR is

dependent on salicylic acid (SA) as a signal molecule and is characterized by increased levels of SA and systemic induction of a set of pathogenesis-related (PR) proteins, the best known being PR-1, PR-2 and PR-5 (Hammerschmidt 2009). SAR is effective against a broad range of biotrophic or hemibiotrophic pathogens, but is not as effective towards necrotizing pathogens. In contrast, the jasmonic acid/ethylene (JA/ET) dependent defence pathway is effective against a broad spectrum of pathogens, including necrogenic fungi. It is associated with the systemic upregulation of PR proteins PR-3, PR-4, PDF1.2, chitinases, chitin-binding proteins and defencins (Ellis and Turner 2001). In addition to the defence reaction, ET and JA are involved in plant development.

Non-pathogenic bacteria have been long known to induce systemic resistance in plants, which is referred to as induced systemic resistance (ISR). ISR can be SA-independent and -dependent and it is partially overlapping with the JA/ET pathway (e.g. van der Ent et al. 2009). ISR is effective against fungal, but also against bacterial pathogens. Unlike the SAR or JA/ET dependent defence pathway, ISR activation does not lead to a massive upregulation of defence network. When Verhagen et al. (2004) screened - by microarray analysis - ISR-induced genes upon treatment of *A. thaliana* by ISR-inducing *P. fluorescens* WCS417r, they found 97 upregulated genes in roots, but no differential regulation in shoots. However, upon subsequent challenge of the plant by plant-pathogenic *P. syringae* pv *tomato*, 81 genes were found to be upregulated in shoots in plants pretreated with *P. fluorescens* WCS417r. Thus, the plants were primed to respond to pathogen attack by ISR (Verhagen et al. 2004).

ISR seems to involve both the SA and JA/ET pathways, as Niu et al. (2011) showed that *Bacillus cereus* AR156 triggered ISR in *Arabidopsis* by simultaneously activating the SA- and JA/ET-signaling pathways and associated marker genes, leading to an additive effect on the level of induced protection. Similarly, Conn et al. (2008) showed that inoculation of *A. thaliana* with endophytic actinobacteria resulted in a moderate upregulation of both defence pathways, and protected the plants against subsequent challenge posed by inoculation with nectotrophic bacterial (*Erwinia carotovora*) or fungal (*Fusarium oxysporum*) pathogens. Although endophyte treatment increased the resistance against both pathogen types, the primed defence pathways differed. Resistance towards *Erwinia carotovora* required the JA/ET pathway, whereas resistance towards *F. oxysporum* was dependent upon SAR. Thus, endophytic bacteria were able to prime both pathways and confer resistance (Conn et al. 2008). Significantly, different *Streptomyces* strains, that (based on 16S sequence and morphology) were closely related, induced and primed

different pathways, as follows: *Streptomyces* sp. EN27 primarily activated the SA-dependent pathway, whereas *Streptomyces* sp. EN28 resulted in enhanced induction of the JA/ET pathway. This might be due to different secondary product profiles of these two organisms. Endophytic *Streptomyces* spp. upregulated, albeit moderately, the respective defence pathways upon ISR induction, unlike rhizobacteria. However, as one of the studied isolates, *Micromonorspora* sp. EN43 did not induce PR genes, but was still able to prime *A. thaliana* upon challenge inoculation, the defence gene induction was not necessary for SR. Rather, the authors speculated that the moderate PR activation observed was due to the *Streptomyces* spp. being detected by plants as minor pathogens. Similar observations were reported in interactions between the endophytic *Arthrobacter* sp. EZB4 and *Bacillus* sp. EZB8 and pepper, where endophyte inoculation resulted in increased proline levels indicating biotic stress. However, despite the initial (mild) stress, these endophytes increased plant biomass and protected them against abiotic stress (Sziderics et al. 2007).

At the cellular level, ISR induction has been studied by Benhamou et al. (2000), who evaluated the effects of ISR induced by endophytic *Serratia plymuthica* strain R1GC4 in cucumber (*Cucumis sativus*) seedlings against *Pythium ultimum.* Seedling treatment with *S. plymuthica* resulted in decreased disease development. Moreover, in endophyte-inoculated plants, fungal colonization was limited to the outermost root layer and deposition of enlarged callose-enriched wall appositions was visible at sites of potential pathogen penetration. Fungal hyphae surrounded by plant-derived deposits were partially disorganized and sometimes disintegrated.

Although most PGPB possess multiple PGP properties, and have simultaneous potential to enhance plant growth and incite disease resistance, the interactions seem to be bacterium/plant specific and complex. For instance, Pavlo et al. (2011) tested plant growth enhancement and defence induction towards bacterial pathogens by two potato endophytes: *Pseudomonas putida* strain IMBG294 and *Methylobacterium* sp. strain IMBG290. *P. putida* was able to protect potato against *Pectobacterium atrosepticum* and also enhanced shoot growth, but *Methylobacterium* sp. was effective in biocontrol only at an inoculum density of 10^5 CFU ml^{-1}, whereas higher inoculum levels led to ineffective disease control or even disease enhancement. In contrast, enhancement of potato shoot growth was only achieved by inoculum densities of 10^6 CFU ml^{-1}. Shi et al. (2011) evaluated *P. putida* MGY2 against papaya antrachnose, a postharvest disease caused by *Colleotrichum glueosporioidus*. They showed that MGY2-treated papaya fruits had lower disease index, lower disease incidence and lesion diameter. The disease suppression was associated with

less softening and lowered ET production in papaya, making the fruit less vulnerable to infection. Additionally, MGY2-treated fruits had increased phenolics and PAL levels compared to the control, indicating activation of the defence pathway by *P. putida* MGY2 (Shi et al. 2011).

Synergistic interactions

Recent studies have revealed that bacterial endophytes might synergistically interact with their hosts improving plant growth. Such endophytes might capture cell-secreted metabolites and other phytotoxic compounds as energy sources and thus ameliorate environmentally-induced stresses. The uptake of plant carbohydrates might also trigger the production of phytohormones in endophytic bacteria. Thus, this two-bladed sword inciting double fitness, might confer advantages to both partners. For example, in the case of ACC deaminase producing bacteria, intercellular ACC is sequestered and degraded by the bacterial cells to supply these with nitrogen (ammonia) and energy (α-ketobutyrate), without disturbing the nutritional balance of the plant (Glick et al. 2007). Furthermore, by removing ACC, the bacteria reduce the deleterious effect of excess ET, ameliorating plant stress and promoting plant growth. Thus, in this case, both the plant and the bacterium benefit from the process, awarding double fitness under adverse conditions (**Chapter 2**, Hardoim et al. 2008). In the genome analysis of the endophytic bacterium *Enterobacter* sp. 638, a region was identified that encodes the uptake and metabolism of sucrose and the synthesis of volatile organic compounds (VOCs - i.e. acetoin and 2,3- butanediol) (Taghavi et al. 2010). Acetoin and 2,3- butanediol are phytohormones involved in plant growth promotion and ISR (Ryu et al. 2003; 2004). In *Enterobacter* sp. 638, the production of acetoin and 2,3-butanediol was dependent on the presence of sucrose in the growth medium or poplar leaf extracts but not on lactate as the sole carbon source. Furthermore, the transcription of genes involved in the synthesis of acetoin and 2,3-butanediol was induced by the uptake of sucrose. Therefore, the authors suggested that the uptake of sucrose, a major photosynthate in poplar trees, by *Enterobacter* sp. 638, triggers the production of the phytohormones acetoin and 2,3-butanediol promoting plant growth. These results gracefully demonstrate the synergistic interaction between some metabolites of the host plant and the activity of endophytic bacteria.

Concluding remarks and outlook

Gaps in our fundamental knowledge and future prospects

As highlighted above, the currently emerging understanding of the mechanistic aspects of endophytic bacteria acting as beneficial partners of host plants has great potential to aid in designing strategies to substantially improve the growth and health of host plants. This is especially true when the latter have to develop under stressful conditions. Hence, associations of plants with beneficial endophytes can be seen as furnishing highly valuable additions to the "toolbox" of sustainable agriculture. However, at the same time the emerging research data have shown the glimpses of the extreme complexity and unicity of the interactions between any enodphytic bacterium and its host plant. In particular, the coregulation between endo- and rhizospheric bacteria and fungi, their common host plant and the environmental conditions are complex and as yet poorly understood. In the light of the current research findings, it seems that there are no simple "key traits" that make up a successful and host-benefiting endobacterium. Rather, the beneficial effect of endobacterial presence may be the result of many properties that are compatible with, and complementary to, the host plant genotype and phenotype. Moreover, it appears that each combination of beneficial bacterium and host plant is unique and there are no simple rules that govern its functioning.

Moreover, it appears that our current knowledge on the ecology of bacterial endophytes has strong overlaps with our understanding of prominent rhizobacteria as well as bacterial phytopathogens. Even though we did make great progress in the past two decades in describing the diversities and community compositions of bacterial endophytes across plants and even identified a range of beneficial properties that likely play roles, there are limited studies that precisely elucidate the mechanisms involved in plant-endophyte interactions. In addition, the intricacies of the 1:1 or even tripartite interactions, and the dynamics therein, are just beginning to be understood. Different approaches have been applied. For example, proteomics (Chi et al. 2010; Lery et al., 2011; Miche et al. 2006), transcriptomics (Rocha et al. 2007; Wang et al. 2005), metagenomics (**Chapter 7**) as well as metabolomics (Scherling et al., 2009) have been unleashed to study the dynamics of the plant endophytic communities and their interactions.

On the positive side, several bacterial endophyte genomes have been sequenced and this novel information will greatly help us to unravel the mechanisms of the plant-endophyte interactions. At present, in-depth studies on how bacteria become endophytic, where they reside and how they interact with the host plant are sparse and yet they are

needed to improve our knowledge and ultimately exploit it in our quest to meet the increasing food demand in a sustainable agriculture.

Applied aspects of plant-benefical bacterial endophytes

It has become clear from the aforegoing material, that many factors affect the beneficial properties of endophytes. The beneficial properties are often highly bacterium and host plant-specific, which suggests that rewarding effects seen on one host plant cannot be easily extrapolated to any other host. Long et al. (2008) isolated and characterized bacterial endophytes from *Solanum nigrum* and tested the PGP potential of the isolates on *S. nigrum* and the closely-related species *N. attenuata*. The majority of the isolates that were able to promote the growth of *S. nigrum* produced either IAA or ACC deaminase. However, none of the ACC deaminase producing strains were not able to enhance root growth in *N. attenuata*. Moreover, the introduction of another organisms, *Azoarcus* sp. BH72, slightly induced the JA defence response (two proteins were upregulated) in one of two sister lineages of rice, while a sturdy JA defence response was observed on the second rice lineage (Miche et al., 2006). These results suggest an involvement of the plant genome in compatible endophyte-host interactions. Furthermore, the expression profiles induced by potentially beneficial endophytic bacteria are affected by other (microbial) residents of host plants as well (Ait Barka et al. 2000) and likely also by plant health status. Concerning the latter, Sessitsch et al. (2004) reported that the community structures of bacterial endophytes were different in potato plants performing either well or poorly in the field. Furthermore, bacterial species richness was higher in the better performing plants, either because plant performance was related to the presence and activity of beneficial endophytes or because the conditions in the poorly-performing plants were less favourable for sustaining an endophytic bacterial community.

It thus appears that, for practical purposes, we cannot easily extrapolate results obtained with one bacterium/plant system to any other system. Each such system should therefore be regarded as unique and this goes down as far as the strain and cultivar levels. Moreover, the microbial status of the host plant, and thus the soil in which this plant is grown, plays another key role in determining the plant endophyte status and the magnitude of the beneficial effect that is achieved. Finally, to be successful, colonization of the plant by the beneficial endophyte should follow a predictable and regular pattern, which is associated with the beneficial effect seen. In the light of the often unpredictable influences

from e.g. weather or climate, this issue may turn out to pose the greatest challenge to the successful use of beneficial inoculants meant to act as robust endophytes.

This thesis

To push forward the frontiers of knowledge, the endophytic community of rice plants was investigated in this thesis. Rice plant was chosen on basis of its economical, social and environmental importance. More than half of the Earth's populations use rice as daily staple food, providing 21% of global human per capita energy and 15% of per capita protein (as estimated by the International Rice Research Institute - IRRI, www.irri.org). Rice is cultivated in 165 million hectares worldwide (area half of size of the India territory) and in Asia countries, 90% of the rice is grown in small farms (less than one hectare) with low imput of insumes. To keep rice security, IRRI estimates that an additional 8-10 million tons of rice needs to be produced every year with less land and less water, in a more efficient and environmentally-friendly system. Taken into account the aforementioned PGP properties of endophytes, here I present a nearly five-year-study on the factors influencing bacterial diversity and composition of the rice-associated community. I believe that bacteria communities might complement rice production in a sustainable agroecosystem.

Aims

The aims of this study were:

1. to characterize the bacterial communities that inhabit rice,
2. to identify the modes of transmission and invasion that rice bacterial endophytes use,
3. to shed light on the rice-beneficial traits harboured by rice endophytioc bacteria, and
4. to assess the importance of these different mechanisms for beneficial mutualistic relationships.

Hypothesis

Considering the aims of the thesis, I tested the hypothesis that bacteria selected by rice plants competently colonize rice plant internal tissues independently of environmental factors, i.e. plant genotype and plant physiological status.

Research questions

Based on the aforementioned hypothesis and aims of the study, the following research questions were formulated:

1. How do different rice genotypes and agricultural regimes affect the bacterial communities that are associated with rice root tissues?

2. Which types of bacteria are present and represent key endophytes of rice? Are these bacteria capable of promoting plant growth? Are they adapted to respond to host stimuli?
3. How are endophytic bacterial communities established? In other words, how do environmental factors (i.e. soil type, bacterial invasion and water regime) shape or affect the endophytic bacterial communities?
4. What is the major source of rice bacterial endophytes, the soil or the seed? How does this bear on our view of endophyte transmission routes?
5. What are the functional characteristics of key rice root endophytes that may play a beneficial role for rice plant development?

Outline of the thesis

First of all, we review the available literature on bacterial endophytes of plants, placing great emphasis on the benefits gained by plants when hosting bacterial endophytes. We illustrate how plants might use some of their photoassimilates to attract and interact with mutualistic beneficial bacteria and how specific endophytes might establish synergistic interactions that improve the fitness of both plant and endophyte. Finally, we show how bacterial endophytes can improve plant growth by diverse mechanisms (**Chapter 1**).

In **Chapter 2**, we review the literature with an emphasis on the properties of bacterial endophytes in respect of how they interact with plants. We formulate a novel hypothesis on endophyte behaviour, in which the postulated competent endophytes use their metabolic versatility, especially those containing ACC deaminase, to improve plant growth by ameliorating plant stress.

Then, using DNA-based polymerase chain reaction (PCR) followed by denaturing gradient gel electrophoresis (DGGE), we assessed the root-associated bacterial communities in ten rice cultivars and searched for connections to plant genotype, soil type and nutrient use efficiency. Differences in bacterial community structures across the rice cultivars were clearly detected and are presented in **Chapter 3**.

The endophytic community in roots of a selected rice cultivar (denoted APO) was further investigated by isolation and clone library analyses. In addition, we assessed, in a suite of 20 selected endophyte strains, various PGP and plant-adaptive properties, as well as metabolic capacities. The collected data are presented and discussed in **Chapter 4**.

The isolation of rice root endophytes revealed a plethora of as-yet-uncharacterized species. We selected six strains that were affiliated with members of the genus *Enterobacter*, the most abundant genus assessed from the clone library, and thus described two novel species, presented in **Chapter 5**.

To test the hypothesis formulated in **Chapter 2**, I set up a greenhouse experiment where a selection of 18 endophyte strains were inoculated into gamma-irradiated soils. After one week of incubation, rice seedlings were transferred into the soil and allowed to grow for five weeks under different treatments, i.e. soil types, water regime and different bacterial inoculation densities (BIDs). Then, the bacterial communities from the bulk and rhizosphere soil, as well as the root and shoot endospheres, were assessed. To our surprise, only small differences were observed between inoculated and uninoculated plants. The data are extensively discussed in **Chapter 6**.

In **Chapter 7** of this thesis, we assessed the potential function of the endophytes that reside inside the root tissues of rice by metagonomic analysis. The results of the different studies are extensively evaluated and summarized in **Chapter 8**.

Chapter 2

Properties of bacterial endophytes and their proposed role in plant growth[1]

Pablo R. Hardoim, Leo S. van Overbeek, Jan D. van Elsas

Abstract

Bacterial endophytes live inside plants for at least part of their life cycle. Studies of the interaction of endophytes with their host plants and their function within their hosts are important to address the ecological relevance of endophytes. The modulation of ET levels in plants by bacterially produced ACC deaminase is an important trait that allows interference with the physiology of the host plant. Endophytes with this capacity might profit from association with the plant, as colonization is enhanced. In turn, host plants benefit by stress reduction and increased root growth. This mechanism leads to the concept of 'competent' endophytes, defined as endophytes that are equipped with genes important for maintenance of plant-endophyte associations. The ecological role of these endophytes and their relevance for plant growth will be discussed.

[1] Chapter published in *Trends in Microbiology* (2008) **16** (10): 463-471. The original publication is available at doi:10.1016/j.tim.2008.07.008

Bacterial endophytes

Plants are major contributors to the fixation of atmospheric CO_2 on Earth. The energy captured from sunlight allows plants to reduce the carbon contained in CO_2 and to synthesize an almost infinite range of carbonaceous compounds. In this context, the photosynthates represent major sources of carbon, nitrogen and energy for plant-associated heterotrophic microorganisms, in particular bacteria (Vandenkoornhuyse et al., 2007), making plants very attractive as nutrient reservoirs for such bacteria. However, for a vast majority of these bacteria, the interior and even exterior parts of plants remain forbidden territories because antimicrobial compounds, such as terpenoids, benzoxazinone, and particularly flavonoids and isoflavonoids, are produced by plants (mainly roots) (Bais et al., 2006). On the other hand, plants can require the presence of associated bacteria for their growth and establishment in different ecosystems. For example, it is notoriously difficult to culture transplants of different species in the absence of bacteria (Leifert et al., 1989), which hints at a role of bacteria in plant growth. In addition, rhizobia are an example of a highly evolved mutualistic plant-bacterium interaction (Denison & Kiers, 2004). Thus, microbes profit from plants because of the enhanced availability of nutrients, whereas plants can receive benefits from bacterial associates by growth enhancement or stress reduction. Therefore, mutualistic interactions between host plants and associated microorganisms could have emerged as a result of the clear positive selection exerted on these associations (Thrall et al., 2007).

Bacterial endophytes, i.e. bacteria that are present within plants (see Glossary), have been known for more than 120 years. In 1926, endophytic growth was recognized as a particular stage in the life of bacteria, where it is was described as an advanced stage of infection and a close relation with mutualistic symbiosis (Perotti, 1926). Later, endophytes have been defined as microorganisms that could be isolated from surface-sterilized plant organs (Henning & Villforth, 1940). In agronomy, this concept was further broadened to encompass all bacteria that can be isolated from surface-sterilized plant tissues and do not visibly harm host plants (Hallmann et al., 1997). In accordance with their life strategies, bacterial endophytes can be classified as ‘obligate’ or ‘facultative’. Obligate endophytes are strictly dependent on the host plant for their growth and survival, and transmission to other plants occurs vertically or via vectors. Facultative endophytes have a stage in their life cycle in which they exist outside host plants. In the extreme view, bacterial phytopathogens might be included as (facultative or obligate) endophytes, as they often occur in avirulent forms in plants. For example, *Ralstonia solanacearum* biovar 2, which

can survive in water systems, can occur as an endophyte, in an apparently avirulent form, inside tomato plants (van Overbeek et al., 2004). Moreover, *Xylella fastidiosa* is apparently extremely adapted to life within plant tissue, persisting for a long time without causing harm to the plant (Araujo et al., 2002). Given their transmission to other plants via insect vectors, and their apparent recalcitrance to growth outside the plant, organisms such as *X. fastidiosa* might be considered to represent obligate endophytes. Avirulent forms of plant pathogens should thus be regarded as endophytes, whereas virulent forms of these organisms should not be included. The obligatory endophytic lifestyle will not be discussed further in this review, and the focus will be on facultative endophytes.

The life cycle of facultative endophytes can be characterized as biphasic, alternating between plants and the environment (mainly soil). The vast majority of the microorganisms that can thrive inside plants probably have a propensity to this biphasic lifestyle. In fact, the observed microbial diversities inside plants could be explained by the ability of diverse endophytes to enter into and persist in plants (Rosenblueth & Martinez-Romero, 2006). These endophytes often originate from the soil, initially infecting the host plant by colonizing, for instance, the cracks formed in lateral root junctions, and then quickly spreading to the intercellular spaces in the root (Chi et al., 2005). Although other portals of entry into the plant exist, e.g. wounds caused by microbial or nematode phytopathogens, or the stomata found in leaf tissue (McCully, 2001), root cracks are recognized as the main 'hot spots' for bacterial colonization (Sørensen & Sessitsch, 2006) (Fig. 3). Hence, to be ecologically successful, endophytes that infect plants from soil must be competent root colonizers.

Although the presence of bacterial endophytes in plants is variable and occasionally transient (van Overbeek & van Elsas, 2008), they are often capable of eliciting drastic physiological changes that modulate the growth and development of the plant (Conrath et al., 2006). Often these beneficial effects of endophytes are greater than those of many rhizosphere-colonizing bacteria (Pillay & Nowak, 1997), and they might be exacerbated when the plant is growing under stress conditions (Ait Barka et al., 2006). The sequence of events in endophytic colonization of the plant interior is presumably similar, at least in the initial phases, to colonization of plant roots by rhizobacteria (Hallmann et al., 1997). Indeed, bacteria belonging to the so-called 'root-colonizing rhizosphere-competent bacteria', e.g. members of the genera *Pseudomonas* (e.g. *P. fluorescens*), *Azospirillum* (e.g. *A. brasilense*) and *Bacillus,* are often also found as colonizers of the internal tissue of plants (Rosenblueth & Martinez-Romero, 2006; Hallmann & Berg, 2006). However, it is

assumed that endophytes represent specialized members of these groups, which suggests that the endophytic stage represents an evolved bacterial modus vivendi and temporal developmental stage. A suite of environmental and genetic factors is presumed to play a role in allowing a specific bacterium to become endophytic (Reinhold-Hurek & Hurek, 1998). Thus, the endophytic occurrence of particular bacteria is the result of chance factors, determined by the chances of developing roots coming into contact with effective levels of bacteria that can become endophytic, and deterministic factors, determined by the presence of dedicated genetic systems that allow bacterial-plant crosstalk and an active endophytic colonization process. Many soil- or rhizosphere-dwelling bacteria could therefore turn into successful endosphere colonizers if they possess the capacity to deal with the vagaries of their changing surroundings, from the exosphere to the endosphere, in which different tissues (e.g. root epidermal cells versus root cortex tissue) will require different bacterial responses. In the endosphere, modulation of plant physiology by tinkering with the plant ET levels has emerged as a major strategy, as any effect on this plant stress signal has major impacts on the bacterial niche (Iniguez et al., 2005). Thus, how bacteria tinker with plant ET concentrations is key to their ecological success or competence as endophytes. The concept of 'competent endophytes' is proposed here as a way to characterize those bacteria that posses key genetic machinery required for colonizing the endosphere and persisting in it. This in contrast to opportunistic endophytes which are competent rhizosphere colonizers that might become endophytic by coincidentally entering root tissue, but lack genes that are key to their ecological success inside the plant. Moreover, one could distiguish passenger endophytes that, in the absence of any machinery for efficient root colonization or entry, might enter plants purely as a result of chance events. For competent endophytes, both partners in the newly-emerged bacterial-plant association are positively selected as a result of benefits provided to it from the partner.

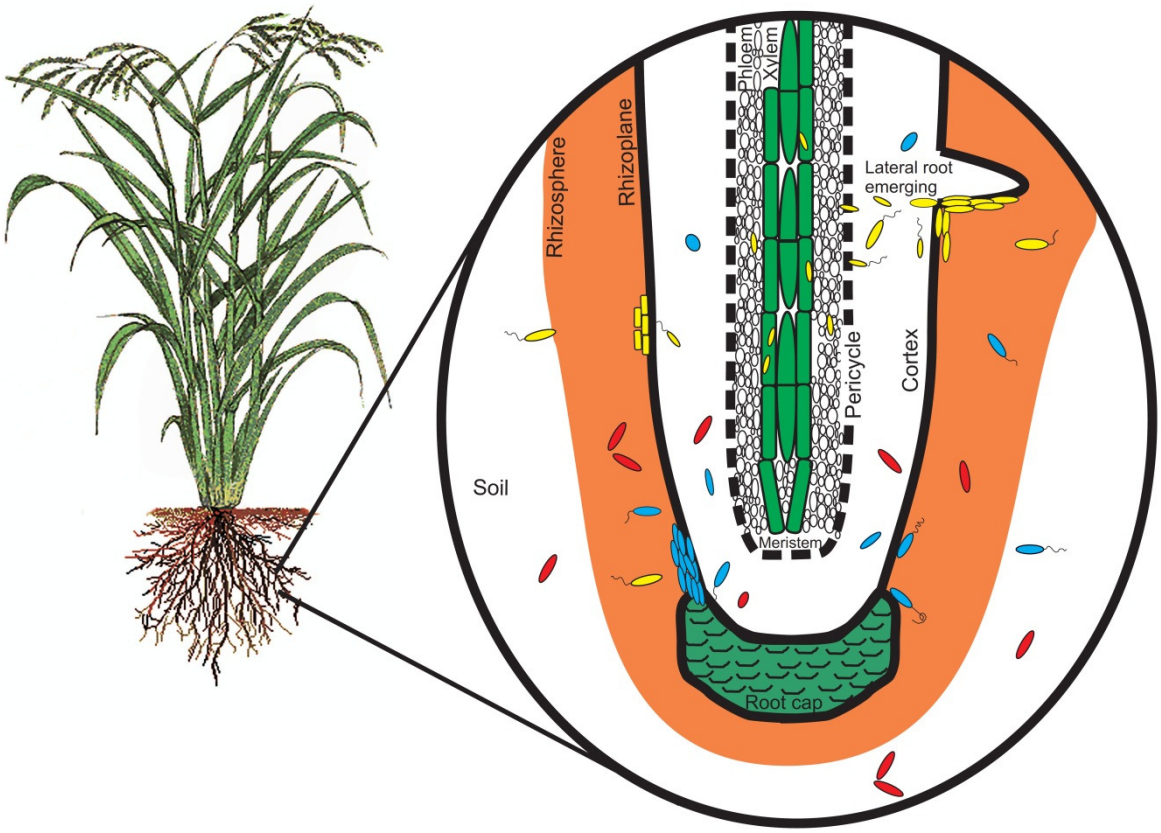


Fig. 3 Types of endophytes and their root colonization process
Stochastic events and deterministic bacterial factors drive colonization of the endosphere, in which a series of events, including microcolony formation at the root surface, is thought to take place. Soil-inhabiting bacteria might become endophytic by chance, e.g. via colonization of natural wounds or following root invasion by nematodes. Such bacteria are considered passenger endophytes (red cells) and are often restricted to the root cortex tissue. Opportunistic endophytes (blue cells) show particular root colonization characteristics, e.g. a chemotactic response, which allows them to colonize the rhizoplane and then invade the internal plant tissues through cracks formed at the sites of lateral root emergence and root tips. However, as occurs with passenger endophytes, opportunistic endophytes are confined to particular plant tissues (e.g. the root cortex). Competent endophytes (yellow cells) are proposed to have all properties of opportunistic endophytes, and, in addition, be well adapted to the plant environment. They are capable of invading specific plant tissue, such as vascular tissue, spreading throughout the plant, and, by tinkering with plant metabolism, maintaining a harmonious balance with the plant host, even when they are present in high density.

The ecology of competent endophytes

The diversity and relative abundance of bacteria in the endosphere is likely governed by stochastic events, which are in turn influenced by deterministic processes of colonization (Battin et al., 2007). Starting from the premise that endophytes commonly originate from the soil in which the host plant is growing, soil factors determine the colonization of plants by different bacteria and, thus, the community composition of bacterial endophytes. Considering the heterogeneity of soil at the microhabitat level and the heterogeneous distribution of plant roots in soil, the early steps in the colonization of plant roots by soil

bacteria likely are truly stochastic events, which depend on the probability with which an effective plant root-bacterium interaction occurs. It has been postulated that this probability will depend on the initial abundance, diversity, physiological status and distribution of putative endophytes in the soil. Factors such as plant genotype, growth stage and physiological status, type of plant tissue, environmental (soil) conditions and agricultural practices will also determine endophytic colonization and endosphere community structures (van Overbeek & van Elsas, 2008; Hallmann & Berg, 2006). Furthermore, intrinsic bacterial traits important for colonization play important roles as determinants of endophyte diversity. For instance, the proportion of isolated bacteria showing swarming (flagellar) motility recovered from the inside of wheat roots was over fivefold higher than that recovered from the corresponding rhizosphere (Czaban et al., 2007). The ability of soil bacteria to approach plant roots via chemotaxis-induced motility and effectively colonize these via attachment and microcolony formation are probably among the strongest deterministic factors that determine the success of bacteria to become endophytic (Bacilio-Jimenez et al., 2003). In addition to traits that confer competence in the rhizosphere, a range of other traits are proposed to make competent endophytes successful in the plant endosphere.

Plant selection for competent endophytes

The bacterial traits involved in the entire plant colonization process are collectively called colonization traits. In the interactive colonization processes, communication between the plant and bacterium (and vice versa) plays a key role (Rosenblueth & Martinez-Romero, 2006) (seleceted bacterial traits described in Table 1). Bacterial root colonization often starts with the recognition of specific compounds in the root exudates by the bacteria (de Weert et al., 2002). These compounds probably also play major roles in belowground community interactions (Bais et al., 2004). Theoretically, plants simultaneously communicate with commensalistic, mutualistic, symbiotic and pathogenic microorganisms via compounds exuded by their roots (Bais et al., 2006). However, it has been suggested that plants can communicate to specifically attract microorganisms for their own ecological and evolutionary benefit (Sørensen & Sessitsch, 2006; Compant et al., 2005a). Due to the complexity of the plant-microbe interactions in soil, it is extremely difficult to understand the detailed mechanisms involved in these putative selection processes. However, lessons can be learned from the well-studied *Rhizobium*-plant interaction, which indicates the

existence of highly-evolved species-specific communication systems (Bais et al., 2006) or from plant-*Pseudomonas* associations, where two distinct plants (flax and tomato) attracted specific 'minority' strains of the *Pseudomonas* species involved, rather than the whole *Pseudomonas* community (Lemanceau et al., 1995). Much like the bacteria selected in the rhizosphere, particular endosphere bacteria might also be selected to establish residence inside plants (van Overbeek & van Elsas, 2008).

Both the *Rhizobium*-legume association (Bais et al., 2006) and the endophytic associations of *Azorhizobium caulinodans*, *Azospirillum brasilense* and *Serratia* spp. with wheat and rice (Balachandar et al., 2006; Webster et al., 1998) involved highly specific (e.g. flavonoid) compounds as signals. The occurrence of chemical communication between bacteria and host plants might suggest that a generic compound or compound class released by plant roots is involved in plant-bacterium interactions. However, there is no evidence for the existence of a single compound, but flavonoids are thought to be generally important in plant-microbe communications (Shaw et al., 2006). They are possibly also important for competent endophytes to occupy a suitable and permanent niche in the rhizosphere and on roots.

Chemotaxis

One factor which strongly contributes to competitiveness in root colonization is the directional motility from the chemotactic response to root exudates (Lugtenberg et al., 2001). This response varies among endophytic species and it is likely that multiple parallel paths evolved during different plant-microbe interactions. Root-exuded organic acids are major chemo-attractants in *P. fluorescens*-tomato interactions (de Weert et al. 2002), while carbohydrates and amino acids attract *Corynebacterium flavescens* and *Bacillus pumilus* to rice (Bacilio-Jimenez et al., 2003). The apparent specificity in these interactions likely relates to bacterial nutritional requirements and in each of these cases, chemotaxis towards specific resources probably determines the specificity of the interaction.

Adaptation to and colonization of the rhizoplane

A competent endophyte, moving towards active zones (zones of enhanced exudation) in the rhizosphere, will gear its metabolism towards a physiological state that allows optimal nutrient acquisition, competition and growth. The organism's environmental sensors will respond to cues that allow the gene expression pattern necessary for colonization. Furthermore, spontaneously occurring variants often show differences in flagellar motility and features important in root colonization, like pyoverdine, cyanide and exoprotease

production, and such phenotypic variations can partially be explained by differences in the *gac*A-*gac*S regulatory system (Haas & Défago, 2005). In the vicinity of roots, the competent endophytes need to attach to the solid root surface (rhizoplane) to reach the primary sites of entry into the roots, i.e. the sites of lateral root emergence, root tips and/or pathogen- or predation-induced wounds. It was recently observed that a *pilT* mutant of the diazotrophic *Azoarcus* sp. BH72 was impaired in its twitching motility, yielding a root-colonization defective phenotype (Bohm et al., 2007). The *pilT* locus plays an important role in the movement of attached bacteria over plant surfaces. Disruption of *pilT* and *pilA*, which are essential for pilus formation and retraction, respectively, reduced bacterial movement, suggesting a key role of these gene products in the colonization capacity of *Azoarcus* sp. BH 72 (Bohm et al., 2007).

When attached to roots, bacteria often increase in numbers by several cell divisions, resulting in the establishment of a microcolony (Compant et al., 2008; James et al., 2002). Invasion of root tissue might take place from such established microcolonies, e.g. at junctions with lateral roots. In this invasive process, enzymatic activity is crucial for degradation of plant cell envelopes. For instance, a functional bacterial endoglucanase gene was important for endophytic colonization by *Azoarcus* sp. BH72 and *Burkholderia* sp. PsJN (Compant et al., 2005b; Reinhold-Hurek et al., 2006). It has been proposed that levels of cell wall degrading enzymes produced by root-colonizing bacteria differentiate bacterial endophytes (low levels) from bacterial phytopathogens (deleteriously high levels) (Elbeltagy et al., 2000). Alternatively, endophytes might enter root or other plant tissue without the aid of cell wall degrading enzymes. Possibly, spontaneously formed cracks between displaced epidermal cells and wounds caused by phytopathogens or soil herbivores constitute important entry portals (Hallmann et al., 1997). Once inside the roots, competent endophytes must pass the casparian strips in the endoderm to systemically spread to the aboveground parts of the plant (McCully, 2001). A determinative characteristic of competent endophytes is that these do not only colonize the plant locally, but are also able to systemically spread throughout the entire plant (Dong et al., 2003; Zakria et al., 2007). It is a challenge to further explore the precise mode of entry, spread and maintenance of these endophytes in the plant root invasion process.

Endophytic colonization

As soon as their cells are inside the plant, competent endophytes will respond to plant cues to allow further induction of cellular processes necessary for entering the endophytic life

stage and spreading to other (intercellular) tissues of the root cortex and beyond. Production of enzymes, such as endoglucanases (Reinhold-Hurek et al., 2006) and endopolygalacturonidases (Compant et al., 2005b), appears to be indispensable in this process. At this point, competent endophytes can quickly multiply inside the plant (Dong et al., 2003; Zakria et al., 2007), often reaching high cell numbers (e.g. 10^8 cells g^{-1} dry weight root tissue) (Barraquio et al., 1997). Endophytic population sizes are dependent on, and positively correlated with, plant developmental stage, progressively increasing from the seedling stage onwards and reaching a maximum (e.g. 10^7 CFU g^{-1} fresh weight (FW) at the senescence stage of potato plants) (van Overbeek & van Elsas, 2008). Also, following the introduction of *Sinorhizobium meliloti* tagged with green fluorescent protein (GFP) into rice and growth under gnotobiotic conditions, the endophyte population densities within the plants were very high, i.e. 9 x 10^{10} cells cm^{-3} of root and leaf tissue (Chi et al., 2005). The reason for the occurrence of such high endophyte densities is not clear, but growth inside plant tissues, rather than continuous invasion is likely involved (Dong et al., 2003).

Plant genes involved in the recognition of beneficial associations with bacteria

The invasion of plants by bacteria could have a major impact on plant growth and health, and plants have evolved molecular mechanisms to deal with the challenges posed by invading bacteria. Many candidate genes with unknown functions have been found to be differentially expressed during plant (sugarcane) – bacterial associations (Rocha et al., 2007). This suggests that the initial steps of endophytic colonization are actively monitored and possibly enhanced or diminished by the plant (Vargas et al., 2003). In particular, the *shr5* gene was differentially expressed after inoculation of sugarcane with specific nitrogen-fixing bacteria that became endophytic (Vinagre et al., 2006). This gene encodes a protein involved in plant signal transduction during establishment of plant-endophyte interactions. Downregulation of *shr5* occurred exclusively when the beneficial bacteria *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and/or *Azospirillum brasilensis* were used. Interestingly, when sugarcane was inoculated with *H. rubrisubalbicans* (an endophytic bacterium which causes mottled stripe disease in a specific genotype), a small decrease in *shr5* gene expression was also observed. Together, these data suggest that sugarcane recognizes beneficial associations with bacteria and responds differently to interactions with non-beneficials. Genes of the ET signaling

pathway were also differentially expressed in the presence of such beneficial endophytes (Cavalcante et al., 2007). Expression of genes encoding ET receptors and the ET-responsive factor was, to some extent, dependent on plant genotype and on the bacterial species used, suggesting that the ET signaling pathway might be involved in the response (Cavalcante et al., 2007).

Modulation of plant ethylene levels

The phytohormone ET is a potent modulator of plant growth and development, playing a central role in plant cellular metabolism (Ping & Boland, 2004). This role can be either positive or negative. ET is involved in the plant developmental cycle, plant disease resistance, microbe-plant interactions and the response to abiotic stresses. In the *Rhizobium*-legume association, the application of exogenous ET, or its direct precursor ACC, inhibits the elongation of infection threads and, consequently, the formation of nodules in most legumes (Sugawara et al., 2006). The endophytic *Klebsiella pneumoniae* strain 342 hypercolonized ET-insensitive mutants of *Medicago trunculata* when compared with the normal plant parental genotype (Iniguez et al., 2005). The addition of an exogenous ET inhibitor, 1-methylcyclopropene, to the parental *M. trunculata* increased the endophytic colonization by *K. pneumoniae* significantly (Iniguez et al., 2005). Furthermore, addition of ACC reduced the endophytic colonization of parental *Medicago sativa* by *K. pneumoniae* 342. These results indicate that ET is a key regulator of the colonization of plant tissue by bacteria and that this regulation is most likely mediated by its effect on the plant signaling pathways. In addition, JA signaling in plant defenses was observed to play a restrictive role in the interaction between the endophyte *Azoarcus* sp. BH72 with rice cultivar IR42, suggesting that it might act independently of ET signaling, restricting endophytic colonization (Miche et al., 2006).

Bacteria are able to modulate plant ET levels by two mechanisms, i.e. by (i) cleaving ACC (Glick et al., 2007) or (ii) inhibiting ACC synthase and/or β-cystathionase, both enzymes of the ET biosynthesis pathway (Sugawara et al., 2006). In both mechanisms, the bacteria will be more efficient at modulating ET levels when they are closer to the plant cells in which ET biosynthesis occurs. Bacterial ACC deaminase, which cleaves ACC into ammonia and α-ketobutyrate, is not currently known to be excreted from the bacterial cytoplasm (Glick et al., 2007). Hence, the decrease of plant ET levels relies on the ability of the ACC deaminase-positive bacteria to take up ACC before its oxidation by the plant

ACC oxidase (Glick et al., 1998). In this context, bacterial endophytes with high locally-induced ACC deaminase activities might be excellent plant growth promoters, as they ameliorate plant stress by efficiently blocking ET production (Cheng et al., 2007). Furthermore, phytohormone-producing bacteria known to stimulate plant growth might even increase plant ET levels (Arshad et al., 2007). To avoid the deleterious effects of ET (e.g. reduced root growth), plants might actually select for ACC deaminase producing bacteria to become endophytic, thereby attenuating plant stress caused by excessive ET levels (Box 1).

Box 1. Modulation of plant ethylene biosynthesis by endophytes

Bacteria with the capacity to synthesize plant hormones, such as IAA, gibberellins (GAs) and CKs, can affect plant growth and development (Saleh-Lakha & Glick, (2006). A phytohormone such as IAA was shown to play an important role not only in plant development but also in activation of the plant defense system (Navarro et al., 2006). IAA might also serve as a signaling molecule in bacterial communication (Spaepen et al., 2007). Furthermore, different IAA biosynthesis pathways could lead to different plant-microbe interactions (Spaepen et al., 2007). For instance, plant-beneficial bacteria produce IAA via the indole-3-pyruvate (IPyA) pathway, while pathogenic bacteria mainly synthesize IAA via the indole-3-acetamide (IAM) pathway (Fig. 4). Considering that excess IAA could result in unbalanced growth (Persello-Cartieaux et al., 2003), the amount of released IAA could play an important role in modulating the plant-microbe association. Furthermore, a member of the ACC synthase gene family, *ACS4*, was transcriptionally induced by IAA in plants, suggesting crosstalk between IAA and ET (Xie et al., 1996). Many aspects of plant life are regulated by ET levels, and the biosynthesis of ET is subjected to tight regulation, involving transcriptional and post-transcriptional factors regulated by environmental cues, including biotic (e.g. pathogenic infection) and abiotic stresses (e.g. wounding, ozone and ultraviolet-B light) (Wang et al., 2002). In the biosynthetic pathway of ET, *S*-adenosyl methionine (SAM) is converted by 1-aminocyclopropane-1-carboxylate synthase (ACS) to ACC, the immediate precursor of ET. This step is probably rate-determining (Broekaert et al., 2006). Thus, the ACS protein plays a major controlling role in ET biosynthesis. In the presence of ACC deaminase-producing bacteria, plant ACC is sequestered and degraded by bacterial cells to supply nitrogen and energy without disturbing the nutritional balance of the plant. Furthermore, by removing ACC, the bacteria reduce the deleterious effect of ET, ameliorating plant stress and promoting plant growth (Glick et al., 2007). Thus, an equilibrium among the plant hormones IAA and ET might be of fundamental importance for maintenance of bacteria inside the plant.

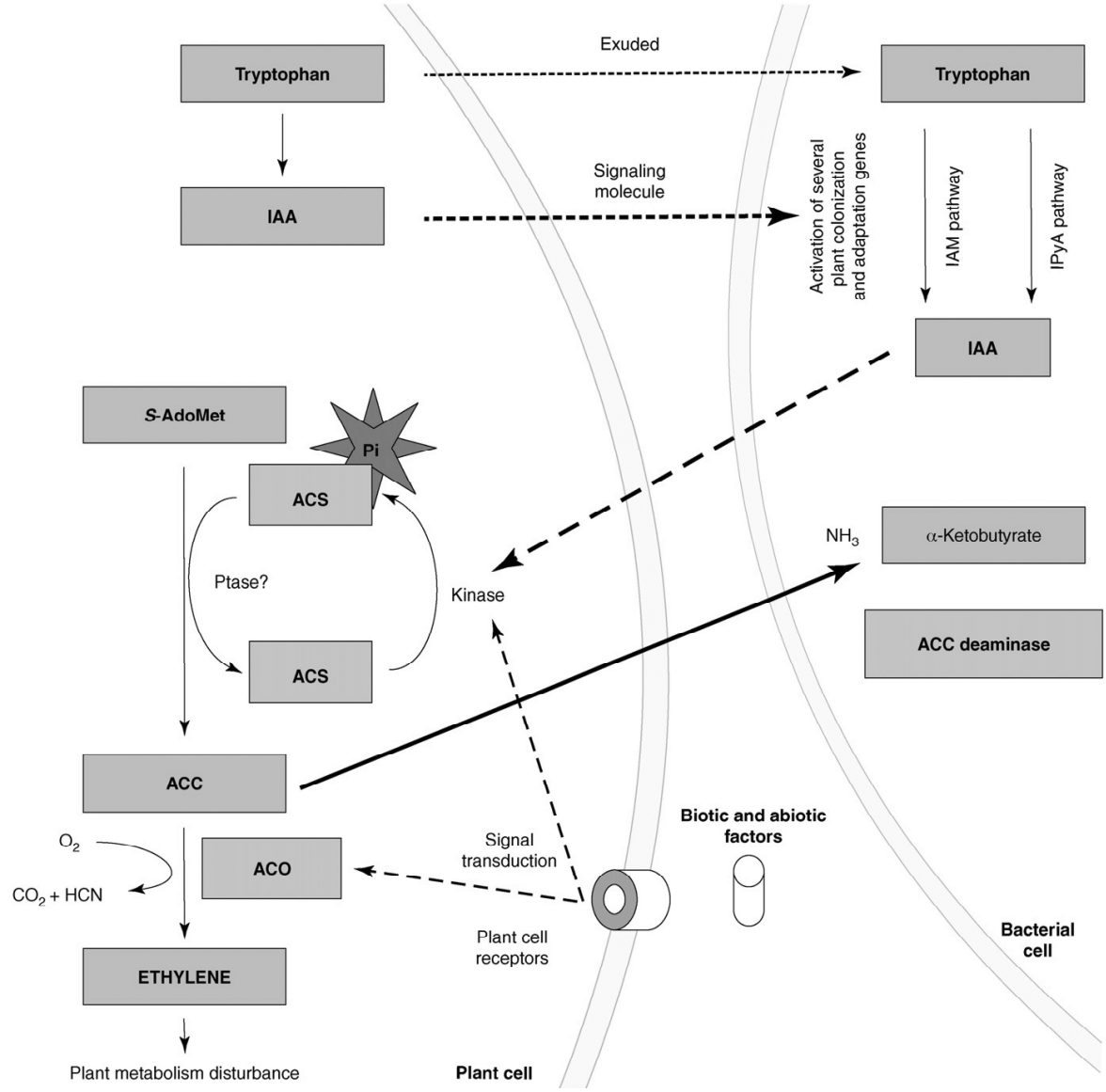


Fig. 4 Schematic representation of plant ethylene modulation by endophytes

Plant-exuded tryptophan is taken up by bacterial cells (dotted arrow) and converted to IAA, mainly via the IAM and IPyA pathways. Plant IAA is also exuded and used as a signal molecule in bacteria (dotted bold arrow). Bacterial IAA is secreted and taken up by plant cells through membrane diffusion and carrier-mediated transport (dashed bold arrow). The multigene family of ACS and ACO (1-aminocyclopropane-1-carboxylate oxydase) is regulated independently by biotic (including auxins) and abiotic factors (dashed arrow). Bacterial and abiotic environmental factors might positively or negatively regulate the plant ET biosynthesis pathway. The phosphorylated form of ACS is more stable and active, converting *S*-AdoMet to ACC. An unknown phosphatase (Ptase) or other mechanism regulates the turnover of the ACS protein from the phosphorylated form (Pi) to the nonphosphorylated form. In the presence of ACC deaminase-producing bacteria, plant ACC is sequestered by bacterial cells and cleaved into ammonia and α-ketobutyrate (bold arrow). In the absence of such bacteria, ACC is oxidized by ACO to form ET, cyanide and carbon dioxide. Figure modified from Ref. (Glick et al., 1998 and Wang et al., 2002).

The selection of such beneficial endophytes might take place at an earlier stage than previously thought. For instance, a high concentration of the phytohormone ET is often needed to 'break' seed dormancy (Kucera et al., 2005). However, once the seed germinates, ET that is still present might inhibit elongation of the root, which is required

for efficient root setting. Hence, plant development is impaired. Colonization by bacteria with high ACC deaminase activities might thus reduce the stress imposed by excessive ET to the plant. The same might occur when plants are subjected to biotic and abiotic stresses (Cheng et al., 2007; Arshad et al., 2007). Preferential selection by plants of bacteria with high ACC deaminase activity (instead of those with low or no activity) could confer benefits to the plant and have been favourably selected by evolution. At the same time, selected bacteria encounter a protective environment in which the supply of nutrients is relatively constant, providing a suitable niche to them. This two-sided mechanism could cause the selected bacteria to be optimally fit as endophytes, thus fitting the concept of competent endophytes. Competent endophytes can thus be characterized by a series of traits that allow them to optimally interact with plant hosts, such as the deamination of ACC, in addition to other traits that enhance their fitness in a plant setting.

Concluding remarks and future perspectives

Our current knowledge of the structure of the bacterial endophytic communities in different plant species is based on both cultivation-dependent and cultivation-independent studies. The combination of both techniques in the same study is recommended because cultivation-based techniques allow the recovery and testing of isolates, whereas cultivation-independent techniques allow the screening for variations in the total endophytic communities (van Overbeek & van Elsas, 2008). Collectively, these studies have indicated that endophytic bacterial communities are dynamic over time, with endophytes showing a development that coincides with plant growth and development. Observed endophytic bacterial communities are often also relatively simple as compared to soil bacterial communities, encompassing tens to up to hundreds of different bacterial types. Hence, it appears that plants can act as true ‘filters’ of soil organisms, selecting those that are successful, competent endophytes (Sessitsch et al., 2002).

Often, the composition of endophytic bacterial communities appears to be rather unpredictable, as considerable variation can be observed even within individuals of the same plant species. Hence, factors that drive the endophytic bacterial communities in terms of the richness and evenness of different types, as well as their nature, from the early stages of colonization of the roots of young plants to the apparently established communities seen in mature plants, are not well understood (Box 2). Intriguingly, there are clear indications in the literature of directional plant selection processes occurring in the phytosphere,

resulting in ecologically relevant plant-bacterium associations (Bais et al., 2006; Rosenblueth & Martinez-Romero, 2006). The importance of assessing the ecological and evolutionary relevance of these processes should be stressed. The enhancement of bacterial colonization spurred by specific carbonaceous exudates by plant roots and the capacity of certain bacteria to modulate plant metabolism are key issues for further study, as these could provide insight into possibly mutualistic plant-endophyte relationships. Particular endophytes could often have important, if not essential, roles for plant growth and development. If such endophytes are not transmitted vertically (e.g. via the seed), then the emergence of efficient physiological systems that allow their selection from soil could have been the key fitness-enhancing traits that enhanced the evolutionary success of these plant species.

Box 2. Outstanding questions

A range of questions about the driving forces and ecological rules behind the prevalence of endophytes within plants remain unanswered. These questions revolve around the poorly understood determinants of the endophytic colonization of plants and the subsequent persistence of endophytes. What are the major factors that shape endophytic communities? How do these communities stabilize and evolve? Do plants indeed preferentially select specific beneficial endophytes, i.e. efficient ACC deaminase producers, from the soil they grow in? Is this an overriding trait that defines the competence of endophytes?

In addition, there are pending questions in respect to the function, beneficial or not, that is exerted by a given endophyte within the endosphere. What function or activity does an endophyte exert to its host plant? How is the balance in plant colonization affected by either a beneficial or a harmful endophyte, i.e. a potential mutualist versus a pathogen? Can plants select for particular groups of plant-beneficial endophytes?

Finally, it is important to understand how plant fitness will be affected by the presence or absence of particular groups of endophytic bacteria. Is the presence of ACC deaminase in bacterial endophytes, which accounts for the effect on plant ET levels, the sole explanatory mechanism? Whereas the deamination of ACC is potentially very important, there is still some unexplained variation in the establishment of endophytes in their host plant. Could this mechanisms allow endophytes to establish outside of their regions of origin (geographically) and could the issue of geography be finally explained (Klironomos, 2002)?

Table 1 Selected bacterial genes involved in colonization and interaction with plants

Class	Function[a]	Gene	Microorganisms	Refs
I Chemotaxis	Motility	*mcp*	*Pseudomonas stutzeri* A15	Rediers et al. (2003)
		cheA	*P. fluorescens* WCS365	de Weert et al. (2002)
II (Exosphere)	Global regulator	*yhbH, hfq, miaA*	*P. stutzeri* A15	Rediers et al. (2003)
	Nutritional adaptation	*pca, pta*	*P. stutzeri* A15	
	Stress adaptation	*bcp*	*P. stutzeri* A15	
	Formation of microcolonies	*pilB*	*Azoarcus* sp. BH72	Dörr et al. (1998)
Colonization	Colony adaptation and DNA rearrangement	*sss*	*P. fluorescens* WCS365	Dekkers et al. (1998)
	Type IV pilus	*pilA*	*Azoarcus* sp. BH72	Bohm et al. (2007)
	Isoflavonoid efflux pump	*ifeA*	*Agrobacterium tumefaciens* 1D1609	Palumbo et al. (1998)
III (Endosphere)	Endoglucanase	*eglA*	*Azoarcus* sp. BH72	Reinhold-Hurek et al. (2006)
	Twitching motility	*pilT*	*Azoarcus* sp. BH72	Bohm et al. (2007)
	AHL-degrading enzyme	*aiiA*	*Bacillus thuringiensis* HD541	Cho et al. (2007b)
	DSF-degrading enzyme	*carAB*	*Pseudomonas* spp. G	Newman et al. (2008)
	Antifungal metabolite	*prnD*	*Burkholderia* spp. ESS4	Mendes et al. (2007)
IV Interactions with plant metabolism	Ethylene modulation	*acdS*	*Pseudomonas putida*	Glick et al. (1994)
		rtxC	*Bradyrhizobium elkanii* USDA94	Sugawara et al. (2006)
	PGP	*alsS*, *alsD*	*Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a	Ryu et al. (2003); Ryu et al. (2004)
	ISR	*srfA*, *pps* operon	*B. subtilis* and *B. amyloliquefaciens* ES-2	Ongena & Jacques (2008); Sun et al., 2006)
	IAA	*ipdC*	Several plant-associated bacteria	Spaepen et al. (2007)
	BNF	*nifH*	*Acetobacter diazotrophicus* PA15, *Azoarcus* sp BH72, *Klebsiella pneumoniae* 342	Rosenblueth & Martinez-Romero (2006)

[a] Abbreviations: BNF, biological nitrogen fixation

Chapter 3

Rice root-associated bacteria – insights in community structures across ten cultivars[1]

Pablo R. Hardoim, Fernando D. Andreote, Barbara Reinhold-Hurek, Angela Sessitsch, Leo S. van Overbeek, Jan D. van Elsas

Abstract

In this study, the effect of plant genotype, soil type and nutrient use efficiency on the composition of different bacterial communities associated with rice roots were investigated. Thus, total bacteria, *Alpha-* and *Beta-proteobacteria*, *Pseudomonas* and *Actinobacteria* were studied using PCR followed by denaturing gradient gel electrophoresis (PCR-DGGE). Rice genotype determined to a large extent the composition of the different bacterial communities across cultivars. Several cultivars belonging to *Oryza sativa* subspecies *indica* tended to select similar bacterial communities, whereas those belonging to subspecies *japonica* and *aromatica* selected ones with divergent community structures. An effect of soil type was pronounced for the *Actinobacteria* communities, while a small effect of 'improved' and 'traditional' plants was noted for all communities analysed. A few dominant bands in PCR-DGGE, affiliated with *Rhizobium radiobacter*, *Dickeya zeae*, *Mycobacterium bolletii* and with members of the *Rhizobiales*, *Rhodospirillaceae* and *Paenibacillaceae* were spread across cultivars. In contrast, a majority of bands (e.g. affiliated with *Enterobacter cloacae* or *Burkholderia kururiensis*) was only present in particular cultivars or was erratically distributed amongst rice replicates. These findings suggested that both bacterial adaptation and plant genotype contribute to the shaping of the dynamic bacterial communities associated with roots of rice plants.

[1] Chapter published in *FEMS Microbiology Ecology* (2011) **77** (1): 154–164. The definitive version is available at www.blackwell-synergy.com

Introduction

Half of the Earth's population depends on rice (*Oryza sativa)* as the source of essential proteins and calories, and hence rice represents an important source of food for mankind (FAO, 2010). The current availability of the sequence of the rice genome, which is small and reveals the presence of genes representative for other gramineous species, has turned rice into a model plant for studies on monocotyledonous plants (Phillips et al., 2007). In particular, the genomes of two rice subspecies (*Oryza sativa* subsp. *indica* and *japonica*) provide rich sources of information on potential functions and interactions during rice crop growth (Cantrell & Reeves, 2002). Despite the advance in rice genome studies, little is known about the putative interactions between rice plants and their associated bacteria (Mano & Morisaki, 2008; Reinhold-Hurek & Hurek, 1998; Ikeda et al., 2007).

Many plant-associated bacteria are involved in processes that affect the plant life cycle, such as fixation of atmospheric N_2, production and modulation of phytohormones and the biocontrol of phytopathogens that frequently affect crops (Rosenblueth & Martínez-Romero, 2006; Compant et al., 2010). These bacteria often interact with their host plants, modulating their physiology and morphology (Feng et al., 2006). For instance, the growth of rice plants has been shown to be promoted by the introduction of specific rhizobia, which enhance growth rates by increasing the water utilization efficiency (Chi et al., 2005). Moreover, the induction of systemic resistance in rice was shown to be triggered by *Methylobacterium* spp. (Madhaiyan et al., 2004a), *Pseudomonas fluorescens* (Nandakumar et al., 2001) and by the harpin protein HpaG from *Xanthomonas oryzae* pv. *oryzicola* (Chen et al,. 2008). *Azoarcus* sp. strain BH72 has been shown to fix N_2 in the aerenchymous tissue of young rice plants (Hurek & Reinhold-Hurek, 2003). Active sulfate-reducing and ammonia-oxidizing bacterial communities have also been found in rice root tissues (Briones et al., 2003; Nicolaisen et al., 2004; Scheid & Stubner, 2001). All of these bacterial activities contribute to, or affect, sustainable rice production.

The make-up of plant-associated bacterial communities is very likely affected by deterministic factors as well as stochastic (neutral) events (**Chapter 2**, Hardoim et al., 2008). Different factors are known to play a role, both qualitatively and quantitatively. The soil, in all of its facets, is one of these factors, because it acts as a major reservoir of bacteria that can colonize the internal tissue of plants (Hallmann et al., 1997). On the other hand, plants offer an environment that is selective to microorganisms (Hallmann & Berg, 2006; Rosenblueth & Martínez-Romero, 2004), "filtering out" specific microbial groups from the diversity found at plant roots. Thus, factors such as plant genotype and

physiological status, bacterial colonization traits, abiotic conditions (e.g. temperature, pH) and agricultural management regimes all can affect the diversity of bacterial communities in root tissues (**Chapter 2**, Hardoim et al., 2008; Van Overbeek and Van Elsas, 2008; Andreote et al., 2010). Among these factors, plant genotype may play a key role in the selection of distinct bacterial communities that associate with plants (Hartmann et al., 2009; Andreote et al., 2009). Although this seems like a simple and easy-to-study phenomenon, so far only diazotrophic communities have been investigated in great detail across various rice cultivars (Knauth et al., 2005; Muthukumarasamy et al., 2007).

In this study, we examined the diversity of bacterial communities associated with rice roots across ten cultivars. All plants except those of the cultivar Moroberekan had been cultivated in the same (flooded) soil under the same agricultural management regime. Our hypothesis was that each cultivar, by virtue of its genetic make-up, selects its own bacterial community from the pool of microorganisms present in the soil.

Material and methods

Field location and sampling procedure

Ten rice cultivars were selected based on:

i) the divergence of the host genotypes, i.e. cultivar Basmati is characterized as *Oryza sativa* subspecies *aromatica*, cultivars Azucena and Moroberekan are *O. sativa* subsp. *tropical japonica* and cultivars DEE, Peta, APO, IR36, IR64, IR65600 and IR72 are *O. sativa* subsp. *indica*;

ii) the soil type where they were cultivated: plants of cultivar Moroberekan were harvested from an upland soil, whereas the others nine cultivars were harvested from a homogenized (rotary spading, once yearly) paddy field;

iii) the response to nutrients, cultivars Azucena, Basmati, Moroberekan, DEE and Peta are denoted as 'traditional' (i.e. low nutrient use efficiency), whereas cultivars APO, IR36, IR64, IR65600 and IR72 are denoted as 'improved' (i.e. high nutrient use efficiency; Peng et al., 2005) (Table 2). Replicate plants of all rice cultivars were sampled from the fields used for rice plant breeding experiments, located at the IRRI (Los Baños, Philippines). For each of the ten cultivars, three individual plants, at minimal distances of 0.5 m from each other, were harvested at flowering stage (Itoh et al., 2005).

Within one hour after sampling, the plants were processed in the laboratory. Therefore, the root mass of each plant was carefully washed under running tap water for removal of adhering soil particles. Root bundles were then separated from the plant aerial tissues using a sterilized scalpel. Selected root parts were further cut into 5-cm pieces and immediately snap-frozen in liquid N_2 and stored at -70 ºC.

DNA extraction from rice cultivars

The snap-frozen root parts from the rice cultivars were subjected to surface sterilization. Briefly, roots were thawed, immersed in 70% ethanol for 2 min, followed by 2% sodium hypochlorite (NaOCl) solution for 2 min, and three successive washes in sterile demineralised water. Such treatment also helps to remove bacterial cells attached to roots surfaces. The surface-sterilized roots (ca 4 g) were transferred to sterile plastic bags containing 2 ml of sterile demineralised water and homogenized by squeezing with a soft-headed hammer in order to release the root-associated/endophytic bacterial cells. Homogenates (400 µl) were directly used for DNA extraction by applying the DNeasy Plant extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with one modification: the cell disruption step was extended from 10 min to 1 h to optimize bacterial lysis. The DNeasy Plant extraction kit has been successfully employed for microbial community studies with vine plants (Dreo et al., 2007; Gambetta et al., 2007) and potato plants (Andreote et al., 2009; Andreote et al, 2010).

PCR amplification of 16S ribosomal RNA (rRNA) genes for denaturing gradient gel electrophoresis (DGGE) analyses

For amplification of 16S rRNA gene regions at total bacterial, nested PCR approaches were applied. The purpose was to suppress the amplification of plant plastid DNA. Thus, primer 799F in combination with universal bacterial primer 1492R was used in the first PCR (Chelius & Triplett, 2001). Each 25 µl PCR mixture contained 1 µl of DNA template (5 - 20 ng), 1x Stoffel buffer, 3.75 mM $MgC1_2$, 200 µM of each dNTP, 400 nM of each primer, 1% formamide, 0.5 mg ml^{-1} bovine serium albumin (BSA), 0.25 µg T4 gene 32 protein (Roche Diagnostics GmbH, Mannheim, Germany) and 2.5 U AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems, Foster City, CA). The thermal cycling conditions and cycle number were as described previously (Chelius & Triplett, 2001). The amplicons were electrophoretically separated in agarose gels (1%), and bands of the expected sizes (ca 740 bp) were excised and extracted using the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions.

Purified amplicons were diluted to final DNA template concentration (5-20 ng) and used in the second (nested) PCR's with primers 968F-GC (carrying a GC clamp at its 5' end) and 1401R-1a (Brons & Van Elsas, 2008). PCR amplifications (50 µl mixes) were performed as described in Brons & Van Elsas (2008). The resulting PCR products were used for DGGE analysis.

To analyze each of the specific bacterial groups, a first PCR specifically targeted the 16S rRNA gene regions of *Alpha-* and *Betaproteobacteria* (Gomes et al., 2001), *Actinobacteria* (Heuer et al., 1997) and *Pseudomonas* (Milling et al., 2004). Each 25 µl reaction mixture for the first PCR amplifications contained 1 µl of DNA template (5 - 20 ng) and PCRs were performed according to their respective protocols (Heuer et al., 1997; Gomes et al., 2001; Milling et al. 2004). The obtained amplicons were used (1 µl) as the templates in the second (nested) PCR as previously described, with the exception of the *Pseudomonas* system, in which the reverse primer 1459R was employed (Milling et al., 2004). All PCRs were carried out in a PTC-200 thermal cycler (MJ Research, Inc., Tilburg, NL).

DGGE profiles and statistical analyses

DGGE analysis was performed in a PhorU-2 apparatus, (Ingeny, Goes, The Netherlands) in 0.5 x Tris-acetate-EDTA (TAE) buffer and gels were run at 100 V for 16 h at 60 ºC. Gel casting was performed as described by Muyzer et al. (2004), with a gradient consisting of 45-65% denaturant (100% denaturant contained 7 M urea and 40% formamide). The amplicons (150 ng) from ten cultivars with 3 individual plants (i.e. replicates) each were loaded side-by-side in the same gradient gel and were cross compared with each other. Reference markers (Garbeva et al., 2001) were loaded at both edges and one in the middle of the gel for normalization purposes. For each PCR-DGGE system (i.e. total bacteria, *Alpha-* and *Beta-proteobacteria*, *Pseudomonas* and *Actinobacteria*) one denaturing gradient gel was used. After the run, gels were stained with SYBR gold (Molecular Probes, Leiden, The Netherlands) and the DGGE patterns were made visible by illumination with ultraviolet (UV). The profiles were digitized using a digital camera and stored as TIFF files.

All PCR-DGGE profiles were analyzed using GelCompar II v 4.06 (Applied Maths, Sint-Martens-Latem, Belgium). After normalization, the position and intensity of individual bands in the profiles (species parameter) were recorded. To assess the complexity of the bacterial communities, bands with similar motility (1% tolerance) were

assigned to the same band migration position. The band intensity and position were used for subsequent redundancy analysis (RDA) using CANOCO 4.5 software (Ter Braak and Šmilauer, 2002). The biplot ordinations were generated by scores of samples (plotted from the ordination of the species data) and effects analyzed (environmental variables). In addition, a Monte Carlo permutation test (with 1000 repetitions) was applied to evaluate the correlation of all microbial communities within the assigned environmental variables (Ter Braak, 1994).

Identification of selected PCR-DGGE bands

Dominant bands from universal, as well as *Alpha* and *Betaproteobacteria* PCR-DGGE profiles were selected for identification. Following excision, the bands were treated for re-amplification, cloning and subsequent sequencing following the methodology described by Costa et al. (2006). In addition, 16S rRNA gene amplicons of identified rice isolates were used to classify PCR-DGGE bands with identical denaturation motility. The sequences from excised PCR-DGGE bands and co-migrated isolates were deposited in the GenBank under the accession numbers HQ702192 to HQ702205.

Results

Analysis of bacterial communities associated with the roots of different rice cultivars

Ten rice cultivars growing at the experimental fields of IRRI were selected at the mature flowering stage (plant height on average 50-70 cm) in accordance with their characteristics (Table 2). Using PCR-DGGE, the compositions of their dominant communities, as related to plant genotype, soil type and nutrient use efficiency, were then investigated. Across the board, total bacterial communities showed the highest complexities with a total of 56 band migration positions; each cultivar (three replicates) containing, on average, 28 bands (Fig. 5a). Communities of *Alphaproteobacteria* were also highly complex, with 52 band migration positions in total and averages of 24 bands per cultivar (Fig. 5b). The communities of *Betaproteobacteria* were intermediate, with totals of 38 migration positions and averages per cultivar of 16 bands (Fig. 5c). Communities of *Actinobacteria* and *Pseudomonas* showed the lowest complexities with, respectively, 19 and 25 total band migration positions and 7.5 bands on average per cultivar for each community.

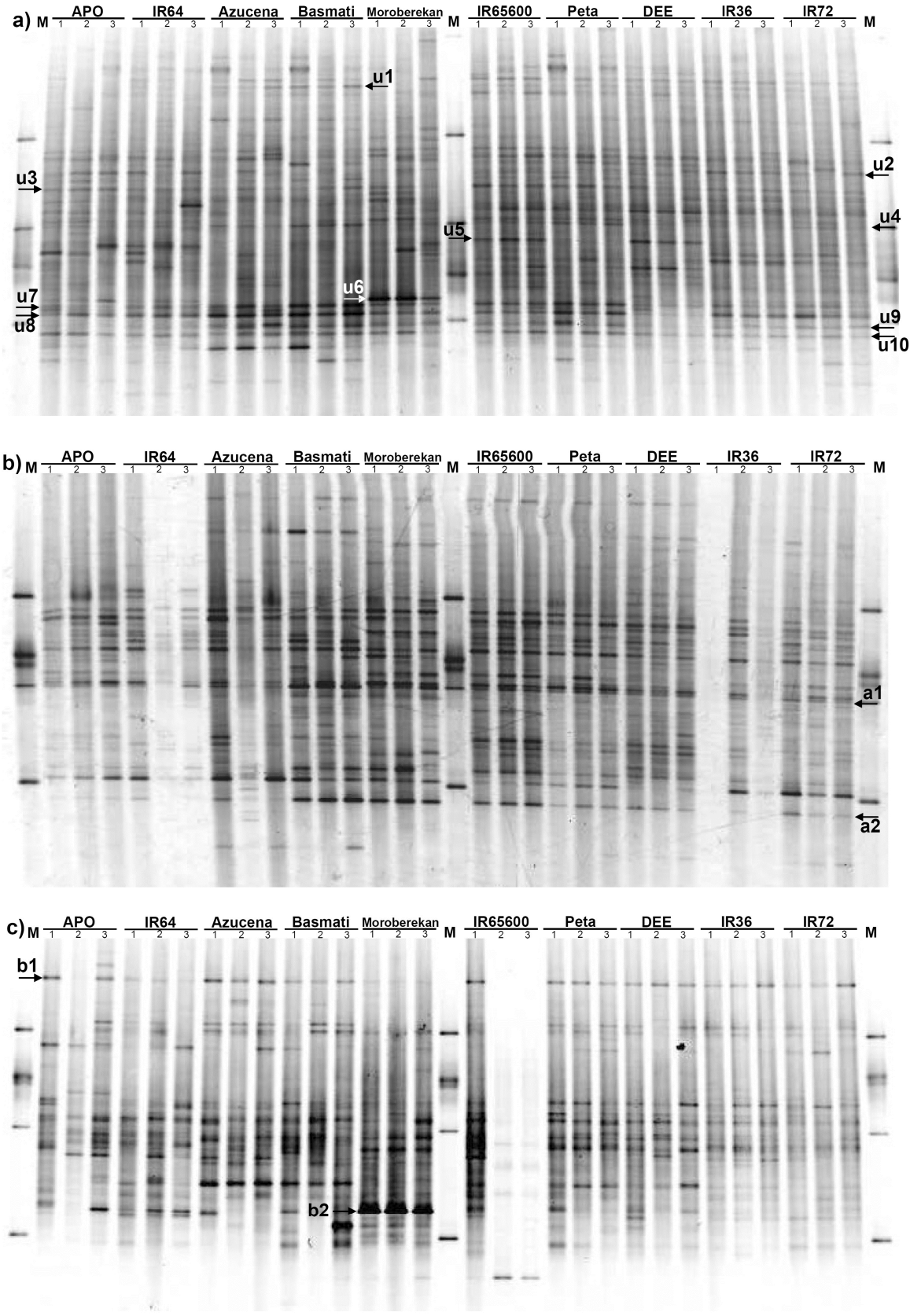


Fig. 5 PCR-DGGE profiles from bacterial (a), *Alpha* (b), *Betaproteobacterial* (c) communities associated with root tissues of ten rice cultivars. Arrows indicate identified DGGE bands obtained from excised bands and co-migrated isolates, respectively, right and left 'head' directions. Internal arrows are cultivar-specific bands, whereas 'edge' arrows are generic bands.

Total bacteria

All bacterial PCR-DGGE profiles contained between 23 and 33 bands within each replicate pattern, suggesting that a considerable bacterial diversity was associated with rice root tissue. Cultivars DEE, Peta, IR65600 and IR72 revealed the highest richness (30 bands), while replicates of cultivars APO, Azucena, Basmati, Moroberekan and IR64 were among the lowest (26 bands). Eight similar dominant bands were present in at least seven of the ten cultivars. From these eight bands, six were identified (Fig. 5a; Table 3). Three showed high 16S rRNA gene sequence similarity (respectively 98.5, 100 and 98.4%) to the type species *Dickeya zeae* CFBP 2052^T (band u3, **HQ702194**), *Rhizobium radiobacter* NCPPB 2437^T (band u4, **HQ702195**) and *Mycobacterium bolletii* CIP 108541^T (band u9, **HQ702200**). In contrast, three bands showed low sequence similarity (respectively, 93.5, 92.4 and 93.2%) to the closest type strains *Azospirillum lipoferum* DSM 1691^T (band u7, **HQ702198**), *Paenibacillus terrae* AM141^T (band u8, **HQ702199**) and *Methylocella silvestris* BL2^T (band u10). DGGE bands u10 (**HQ702201)** and a2 (**HQ702203)** were closely related (99.5% sequence similarity) to *Alphaproteobacterium* strain CCBAU 45397 isolated from root nodules of peanuts in China. Furthermore, a dominant band (u2, **HQ702193**) closely related to *Enterobacter cloacae* ATCC 13047^T (98.4% sequence similarity) was found associated with roots of all replicates of cultivars APO, IR36, IR64, IR65600 and IR72, while it was present but erratically distributed among replicates of cultivars Azucena, DEE and Peta (Fig. 5a). Three cultivar-specific DGGE bands were also observed. A dominant band which was closely related to the 16S rRNA sequence of *Escherichia coli* ATCC 11775^T (100% sequence similarity) was found only on replicates of cultivars IR65600 and DEE (band u5, **HQ702196**), a second band, which was closely related to that of *Burkholderia kururiensis* KP23^T (98.6% sequence similarity) was dominant on the replicates of cultivar Moroberekan, whereas it was faintly present in the replicates of cultivars APO and IR64 (band u6, **HQ702197**; Fig. 5a). A third faint band (u1, **HQ702192**), which was present in the replicates of cultivars Basmati and Azucena, was identified as *Staphylococcus epidermidis* ATCC 14990^T (99.7% sequence similarity; Fig. 5a). The remaining bands (12 to 21) were erratically distributed over the replicate PCR-DGGE patterns.

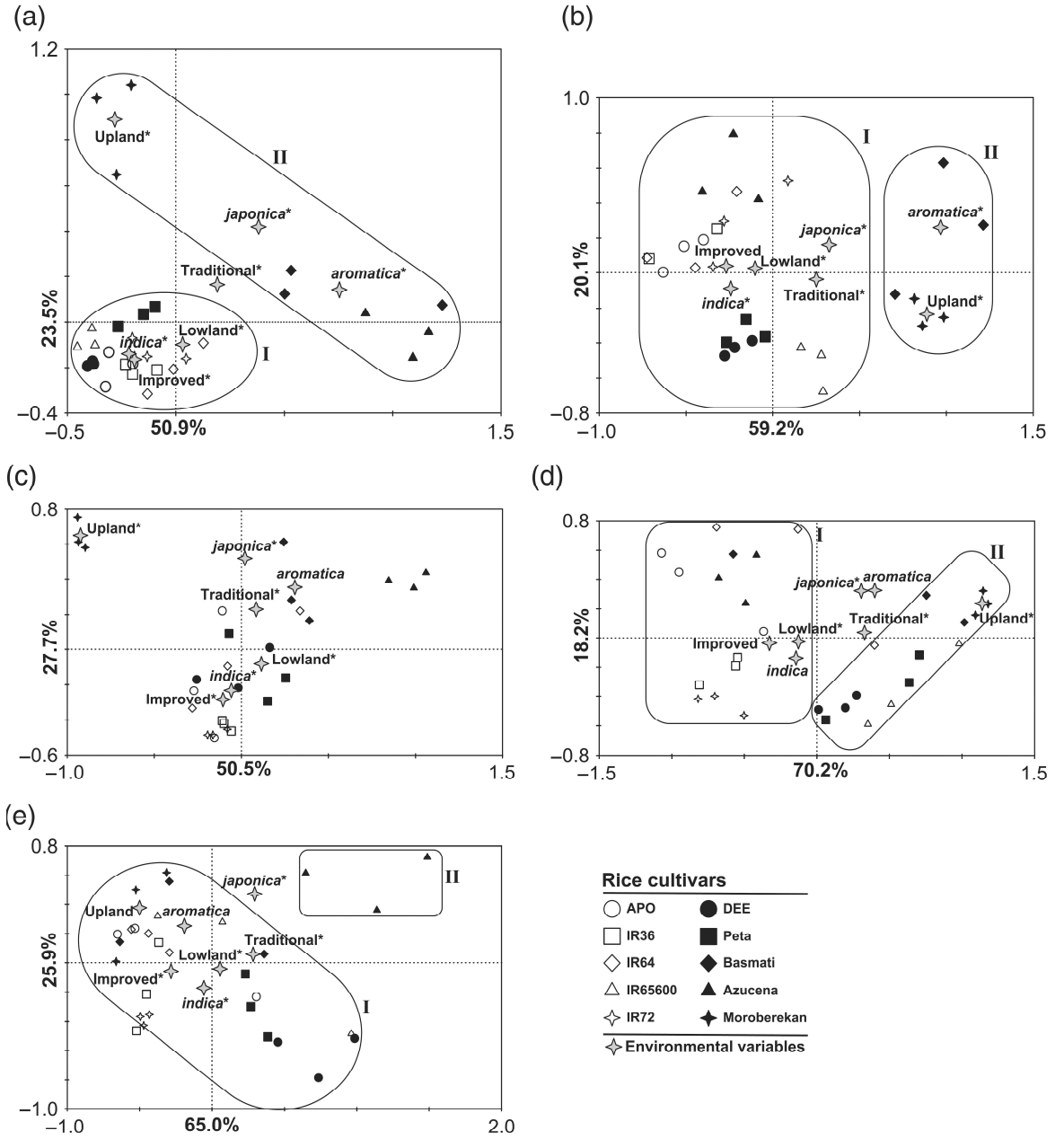


Fig. 6 Biplot ordination diagrams from PCR-DGGE profiles

Retrieved from total bacterial (a), alphaproteobacterial (b), betaproteobacterial (c), actinobacterial (d) and pseudomonads (e) communities associated with roots of ten rice cultivars. Each symbol represents the bacterial community composition associated with roots of an individual plant. Open and close symbols represent improved and traditional cultivars, respectively. Crosses represent the centroid position of the nominal environmental variables (effects). Environmental variable with significant influence on the variation of community composition is shown with asterisks ($P < 0.05$). The variations of each ordination axis are presented in percentage.

Ordination of DGGE patterns

A biplot ordination generated via RDA of the bacterial PCR-DGGE patterns showed a major dichotomy between the total bacterial communities associated with *indica* plants versus those associated with *japonica* and *aromatica* plants (Fig. 6a). Plant genotype

explained most of the variability (48.8%) followed by soil type and nutrient use efficiency with 36.6 and 14.6%, respectively. All effects significantly influenced the variation of the total bacterial composition ($P < 0.05$). Two main clusters, denoted I and II, could thus be distinguished. Cluster I consisted of very similar communities of bacteria exclusively associated with the *indica* cultivars APO, IR36, IR64, IR65600, IR72, DEE and PETA. Cluster II included scattered bacterial communities from two *japonica* and one *aromatica* cultivars, Azucena, Moroberekan and Basmati, respectively. Although the profiles from cluster II were distributed along the second axis, the replicates within cultivars were relatively close to each other, suggesting that, at this level, plant genotype plays a determinative role in the selection of root-associated bacterial communities.

Bacterial group-specific PCR-DGGE analyses

Alphaproteobacteria

The alphaproteobacterial PCR-DGGE patterns showed between 16 and 34 bands across all rice cultivars. The highest richness was found within replicates of cultivars DEE and Peta (average of 28.5 bands), while cultivar IR64 showed the lowest richness, with 16 bands. Ten dominant bands were consistently present in at least eight of the ten cultivars, indicating that these members of the *Alphaproteobacteria* occur in rice roots largely irrespective of plant genotype. Two of these conspicuous bands were identified as *Rhizobium radiobacter* (band a1, **HQ702202**) and *Methylocella silvestris* (band a2, **HQ702203**; Fig. 5b, Table 3), whereas eight remained unidentified. The remaining bands (6 to 24) were erratically distributed across cultivars. A biplot ordination constructed by RDA revealed two main clusters, denoted I and II (Fig. 6b), which were separated along the first axis. Cluster I consisted of patterns of all *indica* cultivars APO, IR36, IR64, IR65600, IR72, DEE, PETA coupled to one *japonica* cultivar (Azucena), while cluster II consisted exclusively of the cultivar Moroberekan and Basmati replicates. The effects plant genotype and soil type significantly influenced the variation in the relative abundance of DGGE bands ($P < 0.05$), where 68.4 and 21.0% of the total variability were explained, respectively. Nutrient use efficiency explained 10.5% of the total variability.

Betaproteobacteria

The betaproteobacterial-specific PCR-DGGE profiles showed considerable variation amongst, and even within, replicates of the same rice cultivars. Totals of 10 to 20 bands were discernable for each of the cultivar replicates (Fig. 5c). Communities of cultivars Peta, Moroberekan and IR36 showed the highest richness, with 18 bands each, while

cultivars Azucena and IR64 had communities with the lowest ones, with 14 bands on average. A conspicuous band (b1, **HQ702204**) found in eight of the nine cultivars analyzed was most closely related to *Uliginosibacterium gangwonense* 5YN10-9^T, at 95.1% 16S rRNA gene sequence similarity. A dominant band (b2, **HQ702205**) occurring in patterns from communities associated with roots of Moroberekan was closest related to *Burkholderia kururiensis* KP23^T, at 98.3% sequence similarity. The remaining bands (8 to 18) were distributed scattered amongst the replicates (Fig. 5c). The RDA ordination diagram revealed a main cluster which consisted of all replicate profiles of the *indica* and *aromatica* cultivars distributed along the second axis (Fig. 6c). Profiles of the two *japonica* cultivars (Moroberekan and Azucena) were adjacent to the main cluster, however opposite to each other. This indicated that the community of *Betaproteobacteria* associated with the roots of *japonica* cultivar Moroberekan differs greatly from those associated with the other *japonica* cultivar Azucena. Plant genotype explained most of the variability (50%), followed by soil type and nutrient use efficiency with, respectively, 38 and 12% of the total variability. All variables, but *aromatica*, significantly influenced the structure composition of betaproteobacterial communities ($P < 0.05$).

Actinobacteria

The actinobacterial PCR-DGGE profiles revealed low richness, with only 4 to 14 bands (Fig. 7a). Three dominant bands were observed across eight out of ten cultivars, while the remaining bands were erratically distributed across cultivar replicates. Although with high variability, two main clusters emerged following ordination by RDA of the PCR-DGGE profiles (Fig. 6d). Cluster I consisted of all replicates of cultivars APO, IR36, IR72 and Azucena, two replicates of IR64 and one of Basmati. Cluster II consisted of all replicates of cultivars DEE, PETA, Moroberekan and IR65600, two replicates of Basmati and one replicate of IR64. The variables lowland and upland significantly differ ($P < 0.05$) from each other and explained 45.7% of the total variation in the ordination. Plant genotype and nutrient use efficiency explained 22.8 and 20% of the total variability, respectively.

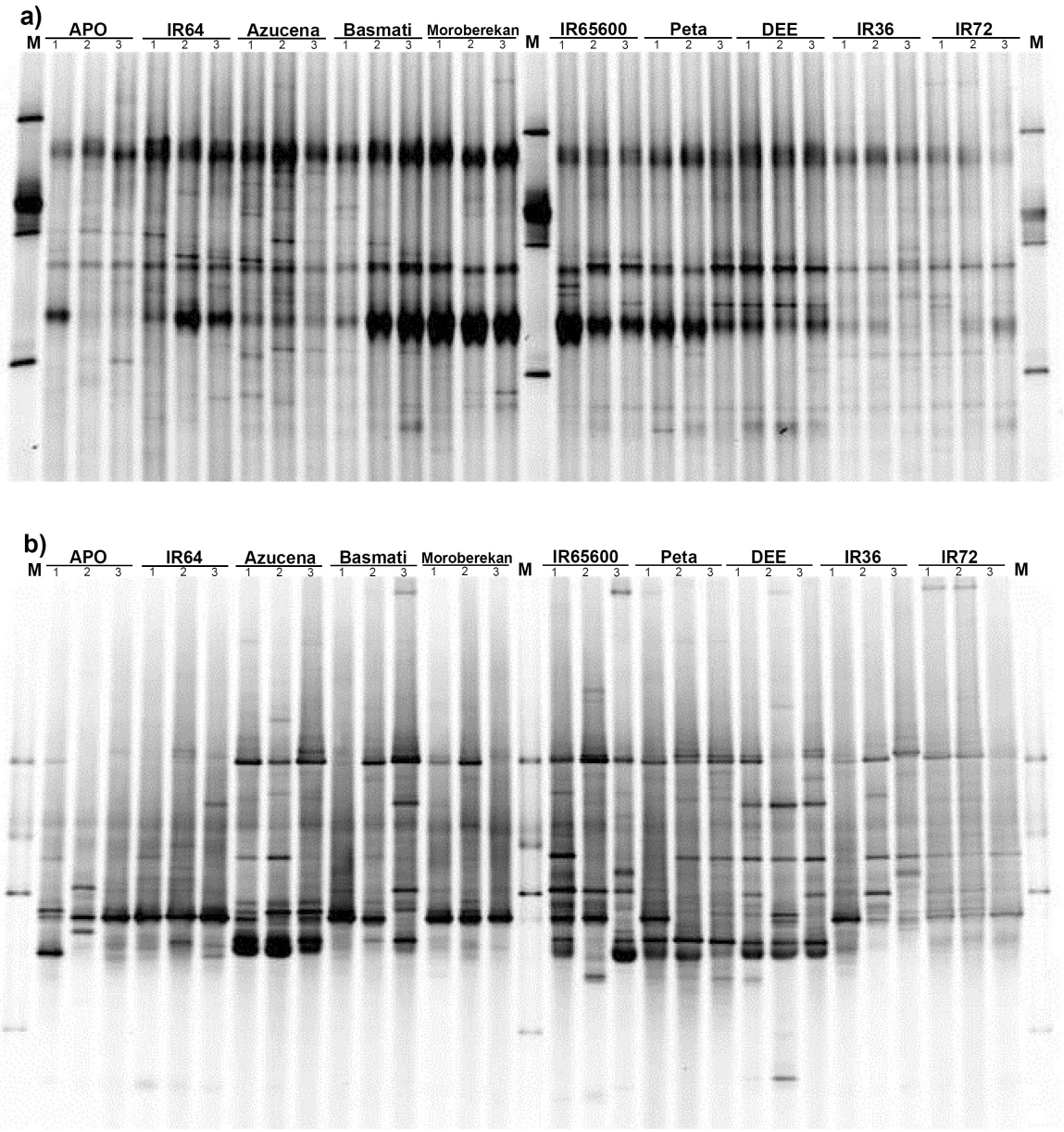


Fig. 7 PCR-DGGE profiles from actinobacterial (a) and pseudomonads (b) communities associated with root tissues of ten rice cultivars

Pseudomonas

The pseudomonad-specific PCR-DGGE profiles consisted of 3 to 11 bands, which were all erratically distributed over cultivars and replicates (Fig. 7b). The highest richness was encountered in the replicates of cultivars Azucena and IR65600, with averages of 10.5 bands per replicate, while cultivars APO, IR36 and Peta revealed the lowest richness, with 6 bands per lane on average. No conspicuous band was observed to occur across six or more cultivars. However, a single conspicuous band was observed in all replicates of cultivars APO, IR36, IR64, IR72 and Moroberekan. Two dominant bands occurred in all replicates of three cultivars; the first one occurred solely in Azucena, Peta and IR65600,

whereas a second band appeared in all replicates of cultivars Azucena, DEE and Peta and was erratically distributed over replicates of cultivars APO and IR65600 (Fig. 7b). RDA ordination revealed two main clusters, denoted I and II, weakly separated regarding plant genotype. Cluster I consisted of pseudomonad communities associated with all *indica* cultivars plus all replicates of cultivars Basmati and Moroberekan (Fig. 6e), whereas cluster II consisted of all replicates of cultivar Azucena. Plant genotype explained 54.1% of the total variation, while nutrient use efficiency and soil type explained 29 and 18.9%, respectively. All variables, but *aromatica* and upland, significantly influenced the structure composition of pseudomonads communities ($P < 0.05$).

Cross comparison over bacterial groups

The roots of *indica* cultivars IR36 and IR72 revealed consistent PCR-DGGE patterns for all bacterial communities analyzed. Moreover, the *indica* cultivars APO and IR64 also revealed consistency for the total bacterial, alphaproteobacterial and pseudomonads communities, but divergence in the profiles of betaproteobacterial and actinobacterial communities. The remaining *indica* cultivars IR65600, DEE and Peta showed large similarity to other *indica* cultivars for total bacterial communities, whereas they formed distinct communities of *Alpha-* and *Beta-proteobacteria* (exception for IR65600) as well as *Actinobacteria*. The two *japonica* cultivars Azucena and Moroberekan revealed great dissimilarity for all communities analyzed, while the bacterial community associated with roots of *aromatica* Basmati resembled both *japonica* cultivars. Cultivar Basmati selected bacterial communities similar to Moroberekan for *Alphaproteobacteria*, *Pseudomonas* and *Actinobacteria*, whereas total and *Betaproteobacteria* were highly similar to the same communities associated with cultivar Azucena.

Discussion

In this study, we present a survey of ten rice cultivars, in which total and group-specific bacterial communities associated with roots were assessed by PCR-DGGE. We were interested in the effect of plant genotype, soil type and nutrient use efficiency on the composition of the selected communities associated with rice and thus examined, at one point in time, ten rice cultivars, of which nine had been grown simultaneously in the same experimental field. Our results indicated that different rice cultivars select specific fractions of the bacterial communities in sometimes highly different and at other times similar fashions. Thus, since the soil habitat, climatic conditions and agricultural practices

were similar across all but one cultivar (Moroberekan), we surmised that the effects seen were for the largest part due to plant cultivar, and, by inference, plant genotype. This observation is corroborated by several other studies in the literature (Sessitsch et al., 2002; Zul et al., 2007; reviewed in Hallmann & Berg, 2006). For instance, in previous studies in rice, the diazotrophic endophytic communities of wild and modern cultivars were shown to be different (i.e. lower diversity in wild cultivars), possibly due to an effect of plant genotype (Elbeltagy et al*.,* 2000; Engelhard et al., 2000). The plant genotype effect most likely results from an effect on rooting and root exudation, thus directly affecting the colonization and nutrient status of bacterial communities associated with roots (Garbeva et al*.,* 2001; Hallmann & Berg, 2006; Bais et al., 2006; van Overbeek *&* van Elsas, 2008).

The communities of total bacteria and *Alphaproteobacteria* revealed considerable complexity, with only a few conspicuous bands being widely distributed across cultivars. This suggested that a deterministic effect of plant genotype leading to highly evolved associations may play a role in the selection of bacterial communities. It is known that many different alphaproteobacterial species can form associations with plants, and such associations may be highly evolved. This is certainly the case for rhizobia, which form N_2-fixing symbioses with legumes, and agrobacteria, which cause phenomena like crown galls on susceptible plants. Strains of *R. radiobacter* (formerly *Agrobacterium tumefaciens*) that do not cause any harm to their hosts are being frequently isolated from root nodules of leguminous plants (Wang et al., 2006). Furthermore, a specific strain of *R. radiobacter* IRBG74, which was isolated from root nodules of the aquatic legume *Sesbania aculeata,* was shown to promote rice growth by enhancing the uptake efficiency of N, P, K and Fe and accumulation IAA on roots of associated plants (Biswas et al., 2000; Tan et al., 2001). The presence of two other conspicuous *Alphaproteobacteria* bands across all cultivars suggests an almost universal adaptation to rice, and one may assume that rice-beneficial functions might be the driving force for their selection.

Members of *Gammaproteobacteria* are important rice colonizers (Mano & Morisaki, 2008). Within this group, *Enterobacteriaceae* species are major players due to their adaptation to broad range of nutritional and physicochemical conditions, as well colonization mechanisms (Holden et al., 2009). The nature of the interactions, being beneficial, deleterious or commensal, may be dictated by the interplay between host and microbe genomes. A conspicuous band (u3), which was present across all rice cultivars, was closely related to the 16S rRNA gene of *Dickeya zeae* CFBP 2052^T (formely *Erwinia chrysanthemi*), a species that contains members that may cause foot rot across Asian rice

(Hussain et al., 2008). Although the sampled rice plants were apparently healthy, the presence of this bacterium may represent a potential threat if conditions lead to disease development. The species *E. coli* encompasses a range of strains that are either commensalistic or pathogenic to animals, and some of them may form associations with varying plant species (Brandl et al., 2006). We are unfamiliar with the *E. coli* type that is behind the band found, but it was recently found that the pathogen *E. coli* O157:H7 can form high population densities on the epidermis and internal plant tissues of alfalfa, reaching up to 10^8 CFU g^{-1} FW within 5 days of inoculation (Teplitski et al., 2009). This may indicate its tight association with these cultivars and possibly relates to the physiology of these. In addition, we isolated, from root tissues of cultivar APO plants, a putative novel species denoted *Enterobacter oryziphilus* REICA_142^T (**Chapter 5**); this organism yielded 16S rRNA amplicons of identical mobility to band u2. The sequence of *E. oryziphilus* REICA_142^T was the most abundant sequence in an extensive 16S rRNA gene clone library generated from the endophytic community of APO root tissues (**Chapter 4**). In addition, *E. oryziphilus* REICA_142^T showed several PGP properties (e.g. fixation of N_2, phosphate solubilisation, ACC deaminase production) as well plant adaptation characteristics (e.g. production of cellulose), which may endow it with the eponymous "competent endophyte" under field conditions.

Three other competent PCR-DGGE bands (bands u8, u9 and b1, respectively), which were spread across most rice cultivars, were assigned to the classes *Firmicutes*, *Actinobacteria* and *Betaproteobacteria.* The members of these classes are often associated with rice plants (Mano & Morisaki, 2008) and can even be isolated from seed tissues (Mano & Morisaki, 2008; Kaga et al., 2009; López-López et al., 2010). Moreover, Moroberekan plants distinctly select for a bacterial species closely related to *B. kururiensis* KP23^T, an N_2-fixing bacterium that is capable of increasing rice biomass via the production of IAA (Mattos et al., 2008). Interestingly, the addition of nitrogen to Hoagland's nutrient solution limited the endophytic colonization of rice by *B. kururiensis* KP23^T in a dose-dependent manner (Mattos et al., 2008), suggesting that agricultural regime has a key role in this interaction.

An effect of soil/agricultural regime was observed for total, alpha-, beta-proteobacterial and actinobacterial communities, as the PCR-DGGE patterns of the cultivar Moroberekan-associated communities differed significantly ($P < 0.05$) from those of the other cultivars. Although Moroberekan plants were sampled from a different soil type and agricultural management regime, the majority of root-associated bacteria were also encounter on other

cultivars, suggesting that rice plants select a restricted range of soil bacteria to form interactions. Both soils (i.e. upland and lowland) have a long history in rice cultivation, thus it might favour the enrichment of rice-adapted bacterial communities. Amongst the bacterial communities studied, only in the *Actinobacteria* PCR-DGGE profile was the majority of the ordination explained by the soil type. One might interpret these findings to indicate that most of the *Actinobacteria* are commonly found in soil as typical soil inhabitants. Thus, factors that relate to the soil, such as those that come about as a result of soil management, may be more determinative than plant genotype (Zul et al., 2007).

Plant breeding strategy targeting high yield crops have an effect on the root-associated bacterial communities. This effect was small for highly complex communities (i.e. total bacteria, *Alpha*- and *Betaproteobacteria*), whereas more prominent in pseudomonads and actinobacterial PCR-DGGE profiles, where 'traditional' and 'improved' cultivars formed distinct bacterial associations. These results support our observation that each bacterial community responds differently (i.e. in a similar or a dissimilar manner) to a specific effect. Our results are in agreement with those of previous studies on bacterial guilds associated with rice cultivars. 'Improved' plants select similar communities of nitrogen-fixing bacteria, which often differ from those of 'traditional' plants (Knauth et al., 2005). In another study, the abundance of ammonia-oxidizing bacteria (AOB) on the roots of different rice cultivars did not differ, however a marked contrast in their population structure was observed, being that 'improved' plants clearly selected for *Nitrosomonas* spp. (Briones et al.,2003).

This study provides new insight in the community structures of bacteria that live in association with rice roots across a range of rice cultivars. We showed that key parts of the bacterial communities respond differently to different plant genotypes. The presence of cultivar-specific phylotypes as well as a few phylotypes that occurred across cultivars emphasized that deterministic factors in accordance with "long-term" interactions contributed to the formation of bacterial communities associated with rice roots. Further studies are needed to understand the mechanisms of the interactions between rice roots and the root-associated bacteria, with a focus on the functioning of these communities in terms of their close interconnections with rice roots.

Table 2 Characteristics of ten rice cultivars used to assess the bacterial community associated with roots

	Cultivars	*O. sativa* subspecies	Observations	Sampled	Origin[a]
Improved	APO	*indica*	Good performance under aerobic conditions and responsiveness to nutrients	Wetland	PI
	IR 36	*indica*	High yield crop, resistance to many insect pets and plant diseases	Wetland	PI
	IR 64	*indica*	Replace cultivar IR 36 as the largest planted cultivar in the 1980s	Wetland	PI
	IR 65600	*indica*	An elite breeding line of New Plant Type (NPT) developed by crossing subspecies *indica* with *tropical japonica*	Wetland	PI
	IR 72	*indica*	Replace cultivar IR 64 in the 1990s	Wetland	PI
Traditional	DEE	*indica*	Parent donor of modern cultivars. Spontaneous mutant, with dwarfing gene	Wetland	TW
	Peta	*indica*	Parent donor of modern cultivars, photoperiod insensitivity. Resistance to tungro virus	Wetland	ID
	Basmati	*aromatica*	Superfine grain qualities, distinct aroma. Tolerant to aluminium	Wetland	IN
	Azucena	*tropical japonica*	Aromatic. Tolerant to aluminium	Wetland	PI
	Morobere kan	*tropical japonica*	Resistance to rice blast and tolerant to drought and aluminium	Upland	GN

[a] Countries of origin: PI- Philippines; IN- India; ID- Indonesia; TW- Taiwan; GN- Guinea

Table 3 16S rRNA gene identification of generic and cultivar-specific PCR-DGGE bands, retrieved from universal and group-specific PCR-DGGE profiles

DGGE band ID[a]	Closest match	Similarity (%)	Closest type strain	Similarity (%)	Present in[b]
u1*	**AY741152**|*Staphylococcus epidermidis*|S09	99.7	**D83363**|*Staphylococcus epidermidis*|ATCC 14990^T	99.7	2
u2*	**DQ091238**|*Enterobacter* sp. FL13-2-1	99.7	**AJ251469**|*Enterobacter cloacae*|ATCC 13047^T	98.4	4
u3	**AF373199**|*Dickeya chrysanthemi*|571	98.8	**AF520711**|*Dickeya zeae*|CFBP 2052^T	98.5	10
u4*	**AY504963**|*Rhizobium radiobacter*|CCBAU 65237	100	**D14500**|*Rhizobium radiobacter*| NCPPB 2437^T	100	7
u5	**AB272358**|*Escherichia* sp. IF4	100	**X80725**|*Escherichia coli*|ATCC 11775^T	100	2
u6	**EF397576**|*Burkholderia* sp. ATSB13	99.7	**AB024310**|*Burkholderia kururiensis*|KP23^T	98.6	3
u7	**AB049112**|*Azospirillum* sp. B518	94.0	**GU256441**|*Azospirillum lipoferum*|DSM 1691^T	93.5	10
u8	**AB486660**|Uncultured bacteria	95.2	**AF391124**|*Paenibacillus terrae*|AM141^T	92.4	10
u9*	**AY457082**|*Mycobacterium chelonae*|ATCC 19237	98.7	**AY859681**|*Mycobacterium bolletii*|CIP 108541^T	98.4	8
u10*	**HM107183**|Alphaproteobacterium| CCBAU 45397	99.5	**AJ491847**|*Methylocella silvestris*|BL2^T	93.2	10
a1*	**AY504963**|*Rhizobium radiobacter*|CCBAU 65237	100	**D14500**|*Rhizobium radiobacter*| NCPPB 2437^T	100	10
a2*	**HM107183**|Alphaproteobacterium| CCBAU 45397	99.5	**AJ491847**|*Methylocella silvestris*|BL2^T	93.2	6
b1	**AB531409**|*Collimonas* sp. III-9	94.0	**DQ665916**|*Uliginosibacterium gangwonense*|5YN10-9^T	95.1	10
b2	**EF397576**|*Burkholderia* sp. ATSB13	99.4	**AB024310**|*Burkholderia kururiensis*|KP23^T	98.3	2

[a] PCR-DGGE bands were identified by sequencing the excised bands or by co-migration with known isolate*

[b] Number of ribotypes present in all replicates out of ten cultivars

Chapter 4

Assessment of rice root endophytes and their potential for plant growth promotion

Pablo R. Hardoim, Angela Sessitsch, Barbara Reinhold-Hurek, Leo S. van Overbeek, Jan D. van Elsas

Abstract

In this study, we assessed the prokaryotic community in roots of rice (*Oryza sativa* L.) by culture-dependent and -independent approaches. We isolated and genomically fingerprinted (BOX-PCR) a total of 222 bacterial strains, of which 82 were distinct from others. On the basis of 530 directly-obtained partial 16S rRNA gene sequences, we identified 16 phyla/classes, with *Gammaproteobacteria* being the most abundant class, followed by *Alphaproteobacteria*. Members of the *Alpha-*, *Beta-* and *Gamma-proteobacteria* as well as *Bacilli* were encountered in both approaches, whereas *Epsilon-* and *Delta-proteobacteria*, *Bacteroidetes*, *Fibrobacteres*, *Planctomycetes*, *Nitrospirae*, *Tenericutes*, *Clostridia*, *Negativicutes*, candidatus division TM7, *Cyanobacteria* and *Crenarchaeota* were exclusively identified in the clones. In contrast, *Actinobacteria* were only found as isolates. The genus *Enterobacter* was the most abundant endophytic bacterium identified by both approaches. In addition, genomic fingerprinting revealed that the *Enterobacter*-related strains were the most diverse. Several plant adaptation and PGP properties as well as C utilization efficiency were investigated in selected strains. Catalase activity, siderophore production, ACC deaminase, solubilisation of inorganic phosphate, nitrate reduction, swimming and swarming motility were observed for at least half of the tested strains, whereas IAA production, fixation of N_2, oxidation of methanol, extracellular cellulase, amylase and protease were inherent to specific strains. The C utilization profiles suggested that each strain had its own metabolic role, which was not always shared between related populations.

Introduction

Gramineous plants represent the most abundant plant community on Earth. It has been estimated that, for instance, grasslands covers approximately 40% of the total land area (Gibson, 2009). Besides their roles in atmospheric carbon sequestration (Carvalho et al., 2010), cereal crops (e.g. rice, wheat and maize) form major sources of carbohydrates for mankind. In the majority of Asian countries, rice is the staple food in the daily diet. Therefore, rice is by far the most important cereal crop regarding its nutritional and economic contribution for emerging economies. Increases in demand have been met by increases in production (c.a. 4.5% y^{-1}), mainly due to increases in area of cultivation, yield improvements gained by improved rice hybrids and the use of fertilizers (Smith and Dilday, 2003).

Despite the importance of microorganisms for plant growth, little attention has been paid in plant breeding programs to the potential to maximize the beneficial effects exerted by plant-associated, in particular endophytic, bacteria (Baldani & Baldani, 2005). Given their vast metabolic versatility, bacteria might increase host biomass by enhancing the efficiency to acquire nutrients, by production and modulation of phytohormones and antagonizing pathogenic species. These processes enhance host sustainability. The importance of endophytes for plant growth and development has been demonstrated for many plant species (Taghavi et al., 2009; Puente et al., 2009; Cheng et al. 2007; Ait Barka et al. 2006; Compant et al. 2010 and references therein).

The diversity of bacterial communities associated with rice has so far been mainly investigated by cultivation-dependent approaches (Mano & Morisaki, 2008). Diverse media have been employed for the isolation of endophytic bacteria from wild and cultivated rice cultivars. Thus, members of the *Actinobacteria, Firmicutes, Bacteroidetes* and *Proteobacteria* have been commonly found in rice tissues, the last phylum encompassing *Alpha*-, *Beta*- and *Gamma-proteobacteria* (Adhikari, *et al*., 2001; Mano & Morisaki, 2008; Tian*, et al.*, 2007). Recently, the application of cultivation-independent approaches to the rice endosphere has allowed the detection of bacterial phyla/classes not yet found by previous techniques. Members of the *Delta*- and *Epsilon-proteobacteria*, *Verrucomicrobia*, *Acidobacteria*, *Cyanobacteria*, candidate phylum TM7, *Deinococcus-Thermus* and also *Archaea* have been detected by sequencing clone libraries obtained for the 16S rRNA gene (Sun*, et al.*, 2008). The high diversity of endophytes inside the rice tissues seems to suggest the existence of a complex environment which might support several microbial guilds.

Many factors might affect the community structure of endophytes, much like observed for rhizosphere and rhizoplane bacteria (Lugtenberg & Kamilova, 2009 and references therein). By using PCR-DGGE with universal and selected bacterial primers, we showed in a previous study that plant genotype is a key factor that determines the structure of the bacterial community, while the soil type, in which the rice was grown, mainly affected the *Actinobacteria* community (**Chapter 3**, Hardoim et al., 2011). Other factors, such as plant physiological status / growth stage, the indigenous endophytic community and agricultural management regime have previously been shown to affect the endophytic community in a variety of plant species (**Chapter 2**; Sessitsch et al. 2004; Van Overbeek & Van Elsas, 2008; Taghavi et al., 2005; Hardoim et al., 2008).

In this study, we first assessed the diversity and structure of the endophytic prokaryotic communities sampled from rice roots of one selected cultivar, APO, using culture-dependent and -independent approaches. Then, we selected strains that occurred frequently and were presumably representative of the rice endophytic populations to assess six plant growth-promotion properties and seven plant adaptation traits as well as strain metabolism in the Biolog-supplemented with 95 carbon source phenotype arrays.

Material and Methods

Sampling procedure

Rice (*Oryza sativa* L.) cultivar APO (IR55423-01) was selected to assess the root endophytic diversity and community composition of prokaryotic microorganisms by culture-dependent and -independent approaches. Ten rice plants were harvested at flowering stage (October 2006) from the rice breeding program plot, with N-P-K fertilizer at 90–30–30 kg per ha, located at the IRRI (Los Baños, Philippines). Roots were thoroughly washed with tap water to remove loose soil particles and then aerenchima root tissues were manually selected by removing dead root tissues and lateral roots. The endophytic extraction of prokaryotic and eukaryotic cells was done as described in **Chapter 7**. Endophytic cells were pelleted at 4°C at 15.000 x *g* for 10 min, and stored in liquid nitrogen for clone libraries or kept on ice at 4°C for bacterial isolation.

Assessment of culture endophytes

The root bacterial community of rice cultivar APO was assessed via dilution plating. For isolation, serial tenfold dilutions were prepared from bacterial cell pellets that had been kept on ice for two days, the transfer period needed to bring the samples to Netherlands.

Aliquots (100 µl) were plated onto heterotrophic R2A agar medium (BD - Difco), after which plates were incubated at 28ºC. Colony development on these plates was monitored for 15 days, and culture communities were thus enumerated. A total of 222 randomly-picked isolates were streaked to purity and stored at -80ºC in 20% glycerol. Pure cultures were subjected to genomic profiling based on interspersed repeat sequence-based PCR with primer BOX-A1R (Martin*, et al.*, 1992). Cell extracts obtained via alkaline lysis (Rademaker *et al.*, 2004) were used as sources of DNA templates. The amplifications were carried out as described by Rademaker *et al*. (2004), using the GeneAmp® PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA). The PCR products were subjected to electrophoresis on agarose gels (1.5%). A single isolate was used as standard control to discriminate the variability derived from PCR runs and agarose gel electrophoresis analyses. Following staining by ethidium bromide, the BOX-patterns were illuminated by UV light and the digitized images were further analyzed for clustering using the Pearson correlation coefficient (GelCompar II). Dendrograms were generated using the unweighted pair group method with mathematical averages (UPGMA) algorithm. The standard isolate control revealed that there was an overall variability of 85% possibly derived from PCR and gel electrophoresis. Therefore by using a threshold of 80% cut-off limit we obtained a total of 82 fingerprint types (FPTs). Representatives of each FPT were subjected to genomic DNA extractions by the Wizard Genomic DNA Purification kit (PROMEGA, Madison, WI, USA). Strains were identified by amplifying the 16S rRNA gene with the universal primers 8F and 1492R (Lane, 1991). The sequences were analyzed in an ABI 377 DNA sequencer (Applied Biosystems) using reverse universal primer 1401R-1a (Brons & Van Elsas*,* 2008).

Ribosomal RNA gene analysis from library clones

Clones library were generated from the rice endophyte DNA using universal bacteria 16S rRNA gene primers (Lane, 1991). Amplicons of five replicate reactions were pooled and ligated into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Ligations were then electroporated into ElectroMAX DH10B™ Cells (Invitrogen), plated onto selective medium agar plates, and sequencing was carried out on an ABI PRISM 3730 capillary DNA sequencer (Applied Biosystems) according to the JGI standard protocols (www.jgi.doe.gov). The bi-directional rRNA gene sequence reads (more than 1300bp) from a total of 696 clones were end-paired and trimmed for PCR primer sequences. Putative chimeric as well as plant organelle sequences were identified using Bellerophon (version 3; http://greengenes.lbl.gov) and BlastN algorithm against *O. sativa* database from

National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov), respectively. A total of 16 and 97 sequences, respectively, derived from, chloroplast and mitochondrial DNA of *O. sativa* subsp. *indica*, 2 from genome of *O. sativa* subsp. *japonica* and 51 putative chimeric sequences were identified and removed from further analysis.

Phylogenetic inference, community biostatistics and sequence identification

A total of 530 clone and 82 strain 16S rRNA gene sequences were used for phylogenetic inference of *Bacteria* and *Archaea* using the ARB phylogenetic software package (Ludwig et al., 2004), against the non-redundant Silva database (release 102). Sequences were aligned using the Web-based SINA aligner (http://www.arb-silva.de/aligner). The evolutionary history of 16S rRNA gene sequences from *Gammaproteobacteria*, *Proteobacteria* classes other than *Gamma* and prokaryotic phyla other the *Proteobacteria* were inferred using the Maximum likelihood method, while evolutionary distances were computed using the General Time Reversable model from MEGA 5.0 (Tamura *et al*., 2011).

The 16S rRNA gene sequences from strains and library clones were further compared for abundance-based parameters (i.e. estimated coverage, diversity and richness indices) using mothur (Schloss *et al*., 2009). A similarity matrix was generated using the Kimura 2-parameter algorithm from previous aligned sequences and operational taxonomic units (OTUs) were assigned, respectively, at 99 and 95% similarity for species and genus cut-off level.

Plant growth-promoting properties and bacterial colonization traits

The bacterial PGP properties: N_2 fixation, production of IAA and siderophore, modulation of ET (via ACC deaminase), solubilisation of inorganic phosphate and catalase as well as plant adaptive traits such as denitrification, production of amylase, cellulase, protease, swimming and swarming motility and the ability to grow in methanol as sole C source were investigated in triplicate for selected endophytic strains (n=20). In addition, the ability to metabolize 95 individual C sources was measured in Biolog GN_2 microplate for 16 Gram-negative strains after incubation for 2 d at 28ºC.

Nitrogen-fixing activity - The ability to fix nitrogen was evaluated by (i) PCR specific primers for detection of *nifH* gene, which encodes for the iron protein of nitrogenase (Poly et al., 2001). Amplicons were subjected to sequencing as described (Pereira e Silva et al., 2011) and identified by blasting the translated nucleotides against the non-redundant protein database from NCBI; and (ii) acetylene reduction activity (Elbeltagy et al., 2001),

where isolates growing in semi-solid Rennie modified medium were injected with acetylene gas into the head atmosphere of the screw cap tubes (15 ml) at a final concentration of 10% (vol/vol) and incubated for 1 d at 36°C. ET concentration was measured on a Chrompack gas chromatograph equipped with a flame ionization detector and a Porapack R column (internal diameter, 2.2 mm; length, 1 m; Varian, The Netherlands).

IAA production - Quantification of IAA production was performed on sucrose minimum salt (SMS) medium (Sheng et al., 2008). Briefly, selected strains were incubated in broth SMS medium with and without 200 μg ml^{-1} l-tryptophan (Sigma) for 2 d at 30ºC. A 1 ml aliquot of the supernatant culture was mixed vigorously with 4 ml of Salkowski's reagent and allowed to stand at room temperature for 25 min before measurement of the absorbance at 530 nm (UV-spectrophotometer, UV-1601, Shimadzu, Japan). The IAA concentration in culture was determined by a calibration curve constructed using pure IAA as a standard.

Phosphate solubilisation - The ability to solubilise mineral phosphate was evaluated by (i) formation of visible halo/zone on NBRIP agar medium (Nautiyal, 1999) incubated at 36ºC for four days; and (ii) quantification of acidic phosphatases by the colorimetric *p*-nitrophenyl phosphate method (Gerhardt et al., 1994). A loop full colony of each strain was suspended in saline solution (0.9%) and then bacterial cells were centrifuged (15.000 x *g* for 5 min). For acidic phosphatase activity 0.3 ml of supernatant was added to 0.3 ml of 0.01 M citrate buffer (pH 4.8) containing 0.01 M disodium *p*-nitrophenyl phosphate. The mixture was incubated for 1 h at 37ºC. The reaction was stopped by adding 0.3 ml of 0.04 M glycine buffer (pH 10.5). The colour development was measured at 405 nm absorbance (UV-spectrophotometer, UV-1601, Shimadzu, Japan). Strains positive for acid phosphate solubilisation was further investigated for production of 2-Ketogluconic acid via the formation of cuprous oxide from culture growing on Hayne's broth medium containing 4% potassium gluconate (Gerhardt et al., 1994).

ACC deaminase, methylotrophic and denitrification activity - ACC deaminase and methylotrophic activity (Corpe, 1985) were qualitatively evaluated on two salt media: DF (Penrose et al., 2001) and M9 (Gerhardt et al., 1994) after incubation for 5 d at 30°C. Culture growth observed on agar media supplemented with 5 mM of ACC (Sigma) as a sole N source and 1% methanol as sole C source indicated ACC deaminase and methylotrophic activity, respectively. Methanol oxidation was further evaluated by PCR specific primers for detection of *mxaF* gene, which encodes for the large (α) subunit of

methanol dehydrogenase (McDonald and Murrell, 1997). Denitrification activity was assessed in cultures growing in nitrate as nitrogen source as described by Gerhardt et al. (1994).

Siderophore production - Siderophore synthesis was analyzed as described by Schwyn and Neilands (1987). Briefly, single colony from overnight culture was streaked on dye Chrome Azurol S (CAS) plates and incubated for 5 d at 30°C. The appearance of orange-colour diffusion zones surrounding these colonies was indicative for siderophore production.

Hydrolytic enzymes and catalase activity - The activity of three hydrolytic enzymes cellulase, amylase and protease was screened on M9 salt medium amended with, respectively, carboxy methyl cellulose (5 g l^{-1}), starch (5 g l^{-1}) and skim milk (2 g l^{-1}). Plates were incubated for 4 d at 28 °C. Activity of enzymes was visualized by formation of halo zone near the colonies after staining procedures as described (Männisto & Häggblom, 2006). The ability to scavenge hydrogen peroxide was evaluated by catalase activity (Gerhardt et al., 1994).

Motility - Swimming motility was assessed on freshly grown liquid culture strains under light microscopy, whereas swarming, i.e. spreading over the agar surface, was detected on R2A plates incubated for 3 d at 28ºC.

Results

Endophytic community of rice roots as revealed by phylogenetic analysis

On the basis of its highly developed root structure and high crop yield, cultivar APO was selected for an in-depth analysis of its prokaryotic community. We thus assessed the root endophytic communities of this cultivar by culture-dependent and -independent approaches. First, plating on R2A agar showed a total abundance of 4.5 x 10^{-5} CFU g^{-1} fresh root tissue, from which 222 random isolates were then obtained in purity. From this total, 82 distinct genomic FPTs were found (Fig. 14). Strikingly, around 43% of the FPTs were unique (i.e. showing a unique PCR-BOX type), whereas the most common FPT encompassed 13 isolates (80% cut-off level). These data indicate high genetic diversity within the culturable rice endophytes.

Both strain and clone 16S rRNA gene sequence libraries mainly encompassed sequences of *Gammaproteobacteria* amounting to, respectively, 77.0 and 53.6% of the total library (Fig. 8). *Alphaproteobacteria*, the second most abundant class, encompassed

9.5 and 22.4% of the strain and clone sequences, respectively. Although in relatively low abundance, members of the classes *Betaproteobacteria* (0.5 and 8.5% of the sequences, respectively) and *Bacilli* (7.6 and 1.1%) were also identified. The clade *Actinobacteria* was solely found in the culturable fraction (5.4% of the total), whereas members of the phyla/classes *Deltaproteobacteria, Fibrobacteres*, *Planctomycetes*, *Tenericutes* and Candidatus division TM7 (0.2%), *Nitrospirae* and *Archaea* (0.4%)*, Epsilonproteobacteria* and *Cyanobacteria* (0.6%), *Bacteroidetes* (0.9%), *Clostridia* (4.3%) and *Negativicutes* (6.2%) were exclusively found as rice root endosphere library clones.

An in-depth analysis of the *Gammaproteobacteria* revealed that 54 and 78% of strain and clone sequences was affiliated with those of the *Enterobacteriaceae*, respectively (Fig. 9). Within the *Enterobacteriaceae,* a major clade encompassing 74% of the clone sequences was observed. This clade belonged to the genus *Enterobacter* with *Enterobacter radicincitans* D5/23^{T} (AY563134) and *Enterobacter* sp. CBMB30 (AY683044) as organisms that showed to highest similarities, at 98.8 and 99.4% sequence similarity, respectively. The cv APO-derived population of *Enterobacter*-related strains revealed high genomic diversity with 22 FPTs. Seven of these were very similar to the most abundant *Enterobacter* clade (Fig. 9). Other members of the *Gammaproteobacteria* found in this study were either known to directly interact with plants, for instance the genera *Stenotrophomonas, Xanthomonas, Methylomonas, Methyloccocus, Acinetobacter, Pseudomonas, Shewanella, Rheinheimera, Aeromonas, Dickeya, Pantoea, Cronobacter* and *Klebsiella*, or have not been previously found to be associated with plants, such as *Steroidobacter* and *Tolumonas*.

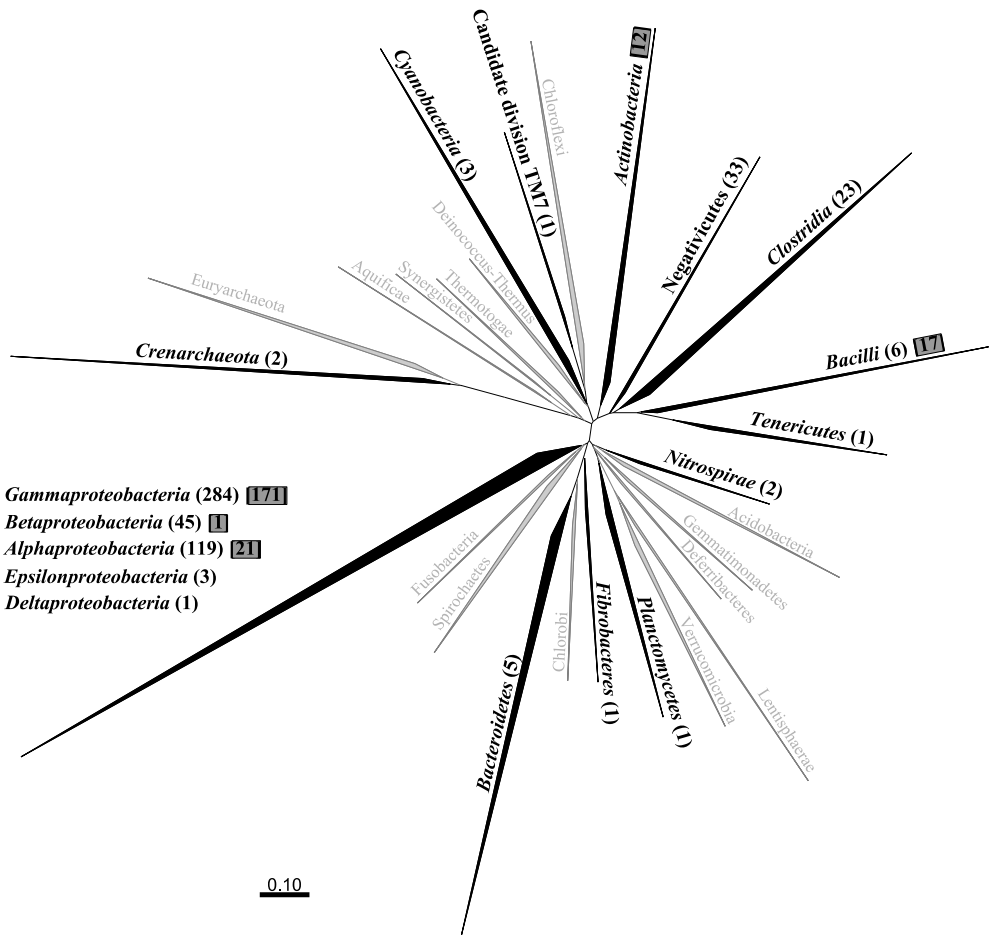


Fig. 8 Phylogenetic analysis of prokaryotic rice endophytes

Neighbor-joining phylogenetic tree of prokaryotic 16S rRNA gene sequences showing the phylogenetic distribution of microbial endophytes detected by 16S rRNA PCR cloning (between brackets) and via isolation (grey highlighted). Sequences were aligned and the tree was constructed using the ARB phylogenetic software package. The PCR-generated clones (530 almost full-length sequences) and isolates (222 partial sequences) contained 16 and 5 phyla/classes, respectively. The bar indicates 10% evolutionary distance.

Among the sequences assigned to the *Alphaproteobacteria*, those affiliated with members of *Sinorhizobium* were most abundant (38% of the total clone sequences), whereas no isolate was obtained from this genus (Fig. 10). A total of 46 clones was very similar to *Sinorhizobium* sp. DAO10 (99% sequence similarity). Another 12 and 8% of the clone sequences, respectively, encompassed *Azospirillum* and *Methylocystis* spp., whereas no isolate was recovered. Members of the genera *Bradyrhizobium* and *Rhizobium* were the most, and second most, abundant with, respectively, 48 and 19% of the total *Alphaproteobacteria* of the isolated strains. In contrast, only 9 and 5% of the clone sequences were assigned to these genera, respectively (Fig. 10).

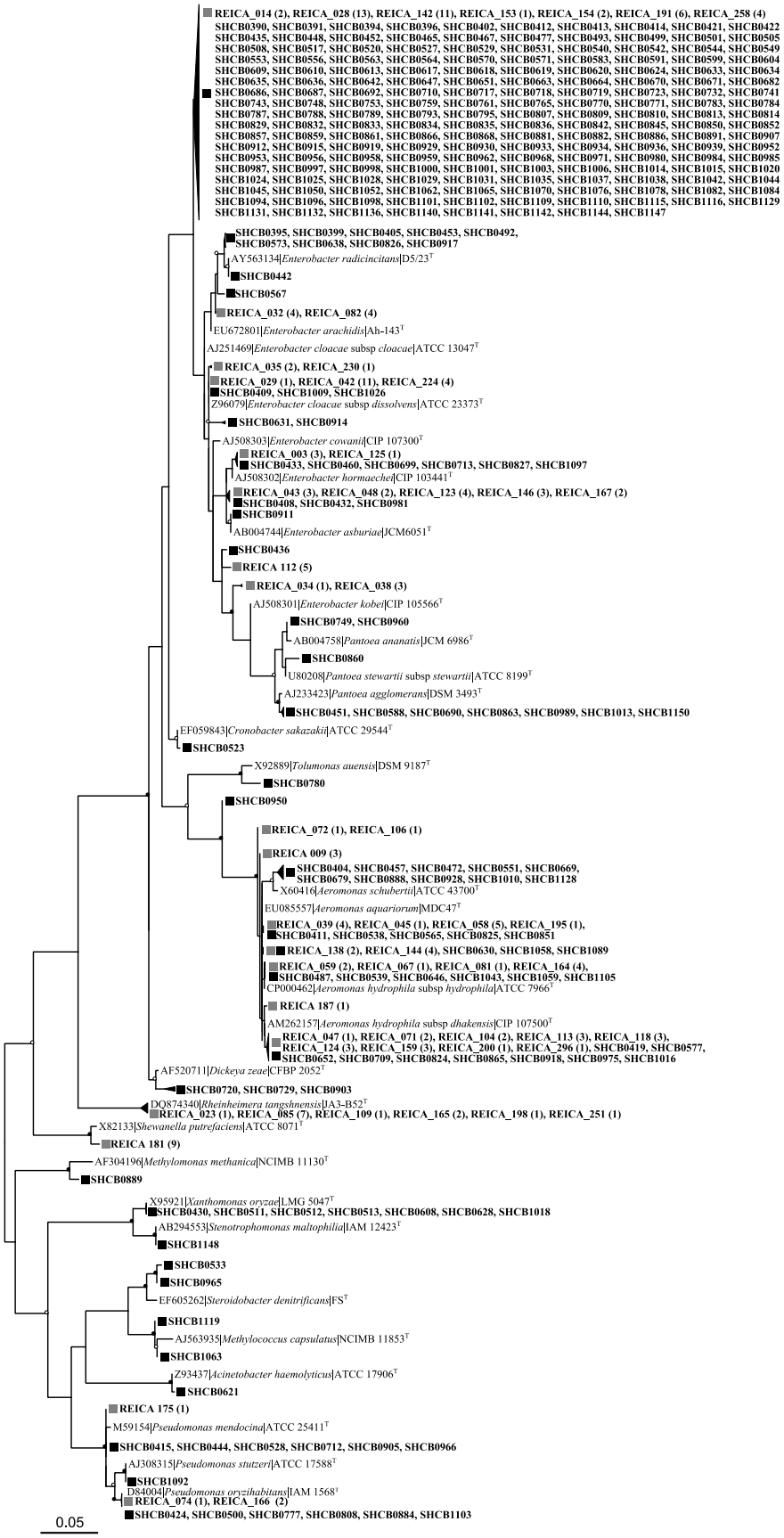


Fig. 9 Phylogenetic analysis of *Gammaproteobacteria*

Phylogenetic analysis of 373 *Gammaproteobacteria* 16S rRNA gene fragments (248 variable positions in a total of 514) retrieved from rice root endophytes of cultivar APO and selected type strains (n=34). The tree branch in which more than 50% but less than 90% and more than 90% of the associated taxa clustered together in the bootstrap test (1000 replicates) is shown in open and close circle, respectively. Rice endophyte isolates (REICA, n=57) and clones (SHCH, n=284) are presented by gray and black square, respectively. The number of isolates with identical FPT is shown between brackets. The scale bar indicates the distance of 5% dissimilarity value among nucleotide sequences.

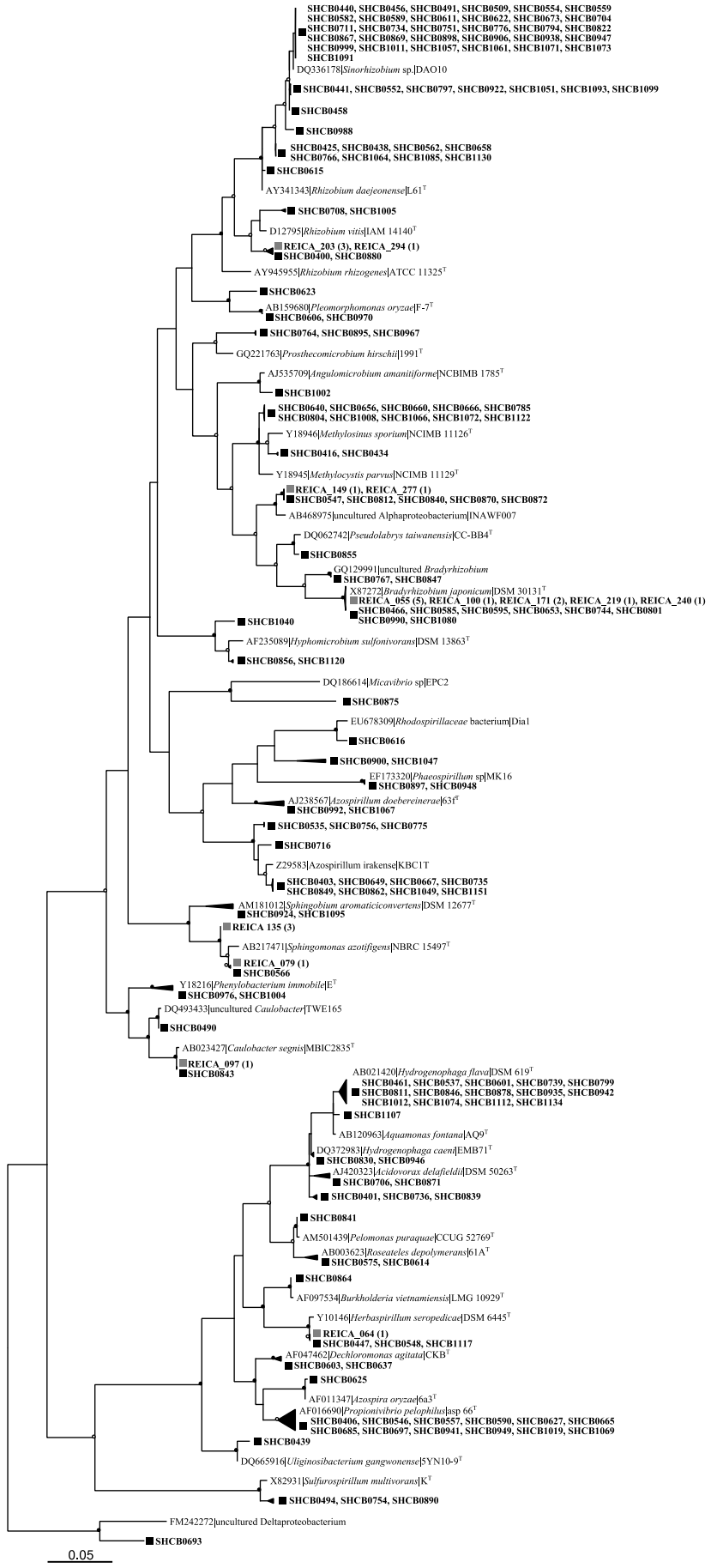


Fig. 10 Phylogenetic analysis of *Alpha-*, *Beta-*, *Delta-* and *Epsilon-proteobacteria*

Phylogenetic analysis of 219 *Alpha-*, *Beta-*, *Delta-* and *Epsilon-proteobacteria* 16S rRNA gene fragments (324 variable positions in a total of 642) retrieved from rice root endophytes of cultivar APO and selected type and reference strains (n=38). The tree branch in which more than 50% but less than 90% and more than 90% of the associated taxa clustered together in the bootstrap test (1000 replicates) is shown, respectively, in open and close circle. Rice endophyte isolates (REICA, n=13) and clones (SHCH, n=168) are presented by gray and black square, respectively. The number of isolates with identical FPT is shown between brackets. The scale bar indicates the distance of 5% dissimilarity value among nucleotide sequences.

Members of the *Betaproteobacteria* were mainly found among the clone sequences, with *Hydrogenophaga* and *Propionivibrio* being represented by, respectively, 31 and 26% of the total abundance (Fig. 10). The only strain found by us from this class (REICA_064) was closely affiliated to *Herbaspirillum* sp. B501 (Y10146; 99.9% sequence similarity). Other *Proteobacteria* that are often plant-associated and were found to belong to the *Beta* subgroup, *Burkholderia vietnamiensis*, *Dechloromonas agitata*, *Azospira oryzae* and *Acidovorax* spp. and to the *Epsilon* subgroup, *Sulfurospirillum multivorans*, were all restricted to sequence clones, which were found in relatively low abundance (less than 5 sequences).

The phylum Firmicutes was the most abundant among the Gram-positive bacteria, accounting for almost 12% of the sequence clones (Fig. 11). Members affiliated to the classes *Negativicutes* (53%), *Clostridia* (37%) and *Bacilli* (10%) were detected in the clone sequences. Strikingly, all 17 isolated strains were restricted to the *Bacilli* (Fig. 11). Within the *Negativicutes*, clones affiliated to the genera *Anaeroacrus* (n=14), *Anaerosinus* (n=6) and *Sporomusa* (n=6) were found. Interestingly, these genera have not been described as being plant-associated. Rather, they were described on the basis of their fermentative metabolism. Similarly, within the *Clostridia* clones affiliated to *Clostridium* spp. (n=22) and *Anaerovorax* (n=1) were found. The remaining clone sequences from these classes were mostly affiliated to sequences of uncultured bacteria or to those of environmental strains (i.e. soil, sediment and freshwater). Within the *Bacilli*, some clone sequences were closely affiliated to *Paenibacillus* sp. (n=3) and to *Bacillus anthracis* (n=3), whereas all (n=17) isolated strains were assigned to *Exiguobacterium acetylicum*. Members of the *Actinobacteria* were also exclusively found as strains, which were closely affiliated to *Mycobacterium* (n=3) and *Micrococcus* (n=8) (Fig. 11). Members of the *Deltaproteobacteria, Bacteriodetes, Fibrobacteres, Planctomycetes, Nitrospirae, Tenericutes, Cyanobacteria,* Candidate division TM7 and *Crenarchaeota* were mainly found among the clone sequences and in relative low abundance (less than 5 sequences; Fig. 11).

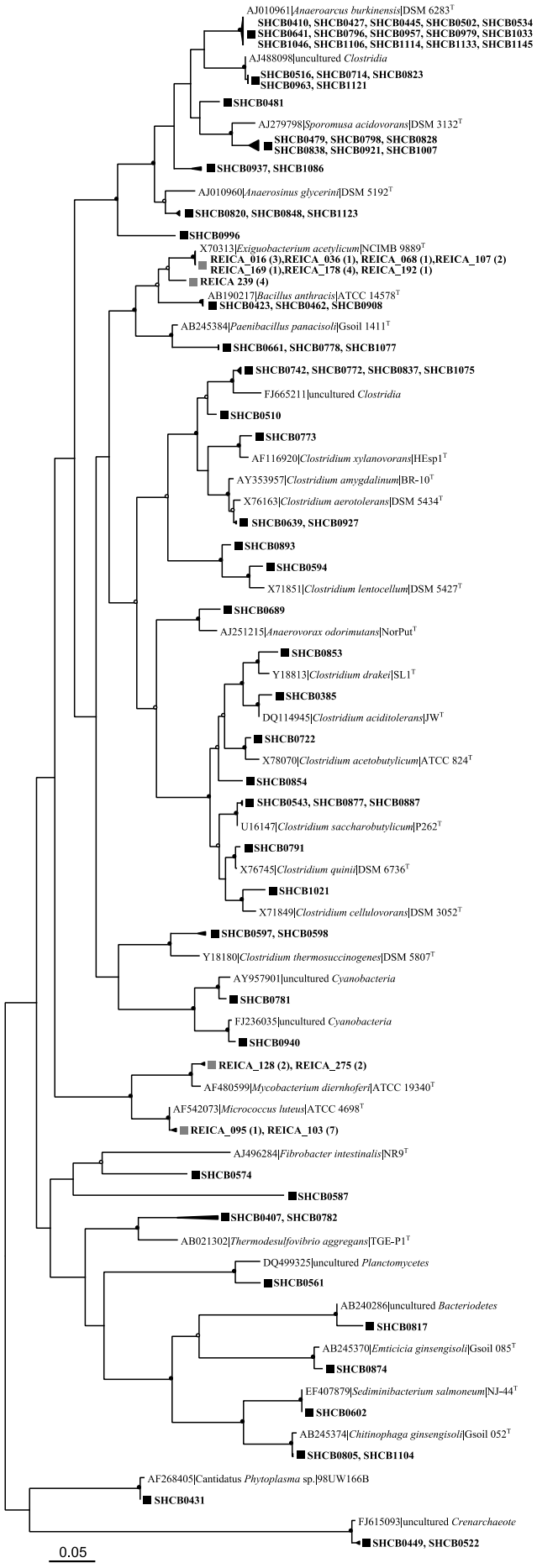


Fig. 11 Detailed phylogenetic analysis of prokaryotic rice endophytes

Phylogenetic analysis of 121 nucleotide sequences of 16S rRNA gene fragments (287 variable positions in a total of 716) retrieved from rice root endophytes of cultivar APO and selected type and reference strains (n=33). The tree branch in which more than 50% but less than 90% and more than 90% of the associated taxa clustered together in the bootstrap test (1000 replicates) is shown in open and close circle, respectively. Rice endophyte isolates (REICA, n=12) and clones (SHCH, n=78) are presented by gray and black square, respectively. The number of isolates with identical FPT is shown between brackets. The scale bar indicates the distance of 5% dissimilarity value among nucleotide sequences.

Community composition and biostatistics

All 82 strain and 530 clone sequences were assigned to OTUs at 99% similarity cut-off for species level assignments (Fig. 12). A total of 196 OTUs were detected, of which 26 and 149 were exclusively assigned to strain and clone sequences, respectively. Within the clones, 113 OTUs were found as singletons. Conversely, 10 of the 82 strains OTUs were singletons. Abundance-based coverage analyses were computed to clone library sequences at cut-off levels of 99 and 95%, which yielded Good's coverage of 79 and 93%. Thus, at the genus level, our clone library assessment nearly exhausted the total estimated richness of root endophytes. The coverage estimations were not extended to the strain sequences.

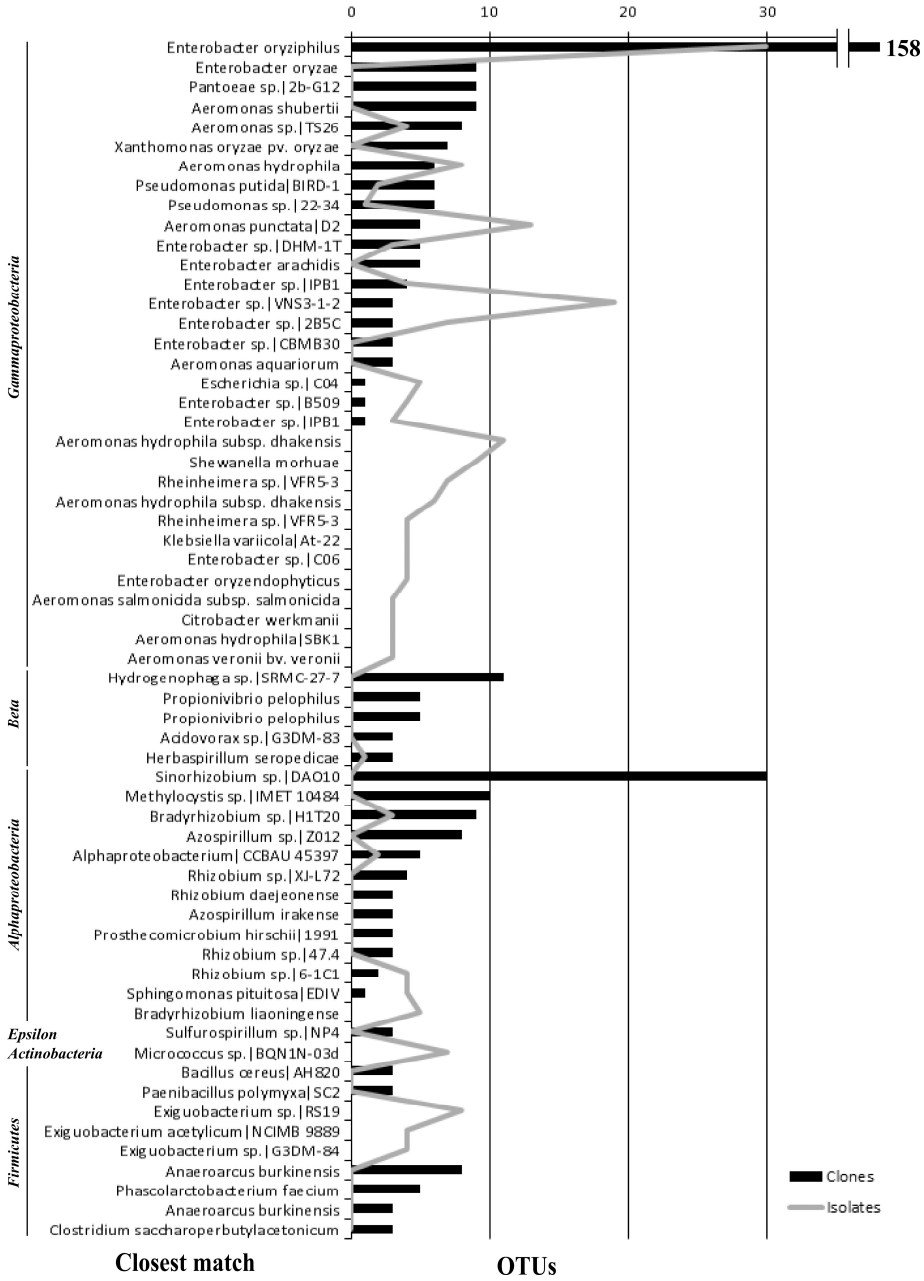


Fig. 12 Distribution of rice endophyte OTUs

Abundance distribution of 44 and 35 OTUs reported on the species level (99% sequence similarity) for, respectively, PCR-generated library and isolate sequences. Only OTU's with more than two sequences in either of both collections (library clones or isolate) are shown.

The isolates were found to represent 10.7% of the total diversity assessed from the clone library. The overlap was higher, i.e. almost 1/3, when only OTUs with more than two clone sequences are considered, suggesting that a great number of endophytes are functional inside the host plant. This was very likely the case for the single most abundant OTU, which encompassed, respectively, 13.5 and 29.8% of all strain and clone sequences and was assigned as *Enterobacter* sp. Members of *Enterobacter* spp., *Aeromonas* spp., *Pseudomonas* spp., *Escherichia* sp. C04, *Herbaspirillum seropedicae*, *Bradyrhizobium* sp. H1T20, *Rhizobium* sp. 6-1C1, uncultured Alphaproteobacterium CCBAU 45397, *Caulobacter segnis* and *Sphingomonas pituitosa* with 99% sequence similarity were found in both approaches (Fig. 12).

Plant growth-promoting, adaptive properties and metabolic profile

Plant growth promotion and adaptation traits were further investigated on selected rice endophytes (n=20; Table 4). One strain for each genus was analyzed, except for *Enterobacter* spp. (n=4), *Bradyrhizobium* and alphaproteobacterium (n=2). Among the strains tested for plant growth promotion activity, 20% showed N_2 fixation capacity, 45% revealed the capacity to synthesize IAA, 50% showed phosphate solubilisation activity via the production of organic acids, 60% showed the ability to grow on ACC as the sole C source, 70% was able to produce and secrete iron-chelating compounds and 80% showed catalase activity. The beneficial activities vary from strain to strain, in which not a unique pattern was observed more than once amongst tested strains (Table 4). The *Enterobacter* sp. REICA_142 and *Rhizobium* sp. REICA_203 revealed the highest nitrogenase acitivity (4.6% of acetylene reduction in 24h) and IAA production (86.5 μg ml^{-1}), respectively. The phosphate solubilisation activity of *Enterobacter* spp. REICA_142, REICA_082 and *Klebsiella* sp. REICA_034 was correlated with the production of 2-Ketogluconic acid, however, the solubilizing activity of the other strains was not. Analysis of the sequences of the translated *nifH* gene from two major *Enterobacter* populations, REICA_142 and REICA_082, showed 100 and 99% amino acid sequence identity to a *nifH* fragment from *Pantoea* sp. A0310 (FJ593774), whereas the *nifH* gene of *Klebsiella* sp. REICA_034 and *Herbaspirillum* sp. REICA_064, showed, respectively, 100% amino acid sequence identity to the *Klebsiella* sp. AL060224_03 (ACM68399) and *Herbaspirillum* sp. B501 (BAD77945). The plant adaptive tests revealed that 10% can oxidize methanol or produce amylase, 15% protease, 40% cellulase, 50 and 60% showed swarming and swimming motility, respectively and 75% revealed partial denitrification (Table 4).

The capacity to utilize 95 sources of C was further investigated in 16 selected Gram-negative strains. The ordination diagram with strains and C sources that differentiate the strain metabolisms revealed four main groups, each occupying one quadrant (Fig. 13). Quadrant I is represented by one strain of each proteobacterial class, whereas quadrants II and IV are exclusively represented by members of the *Alphaproteobacteria* and *Gammaproteobacteria*, respectively. The majority of strains in quadrant III belonged to the *Gammaproteobacteria,* with one *Alphaproteobacteria* (REICA_203).

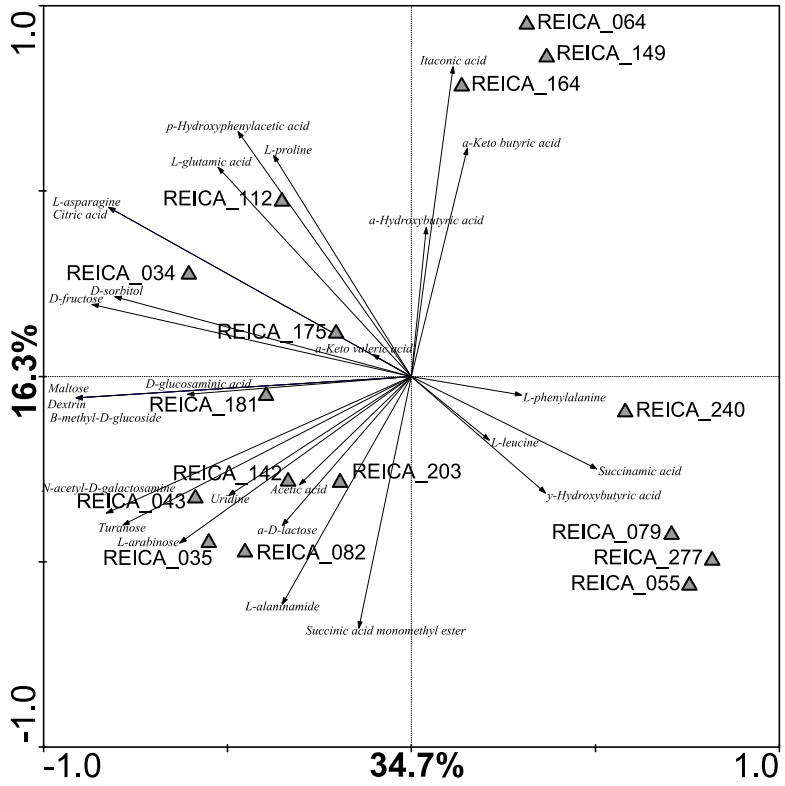


Fig. 13 Metabolic profiling of selected strains

Bi-plot ordination diagram of principal component analysis from 16 rice endophytes describing the carbon utilization capacity (n=95) among them (strain ID see Table 4). Only discriminative carbon usage requirements (n=27), represented by arrows, are shown to facilitate visual interpretation.

Discussion

In this study, we present the most comprehensive assessment of the prokaryotic community retrieved from the endosphere of rice root tissues. Our results revealed that the diversity of endophytes, assessed by culture-dependent and -independent approaches, is much higher than previous observed (Mano & Morisaki, 2008). From both techniques, members of *Proteobacteria* were by far the most abundant assessed phylum. Similar results have been found in the root tissues of various plants (Hallmann & Berg, 2006), including rice (Sun et al., 2008). Furthermore, our isolation procedure on R2A medium

revealed that almost 33% of the total community observed from clone sequences was recovered, suggesting that many endophytes are heterotrophs. One might expect it, once plants synthesize a vast range of photoassimilates; therefore bacteria thriving inside plant tissues must cope with rich source of organic nutrients.

Recent studies have demonstrated that species richness and the so-called "community niche" (van Elsas et al, 2010) are positively correlated. This might explain the better or more robust functioning of a more diverse system as compared to a lower-diversity one. It is plausible that natural selection will thus push such systems to the higher end of the diversity scale (Salles et al., 2009; Langenheder et al., 2010). The multiple metabolic functions of endophytes may, in an overall fashion, be beneficial to the performance of the host plant. Moreover, maintaining the diversity may be cheap for the plant, as it is mainly sustained by the host photoassimilation capacity, which creates a rich source of nutrients for heterotrophic microorganisms to thrive on.

Gammaproteobacteria was the single most abundant class inside rice roots, with *Enterobacter* spp. been the most abundant genus and most genetically diversity community amongst strains. In additional to high diversity, we recovered several strains identical, at 16S rRNA gene sequence, to the most abundant clone. These strains, represented by *Enterobacter* sp. REICA_142 were close assigned to the endophyte *Enterobacter* sp. CBMB30. The strain CBMB30, isolated from rice in South Korea, showed many plant growth promotion capabilities, i.e. the production of auxins and CKs and the fixation of N_2 (Lee et al., 2006), similar to those observed by us. Furthermore, analysis of the sequences of the translated *nifH* gene from *Enterobacter* sp. REICA_142 and *Enterobacter* sp. REICA_082 showed that they were close related to *Pantoea* sp. A0310. *Pantoea* sp. A0310 together with *Klebsiella* sp. AL060224_03, a strain which the *nifH* gene was identical to another rice root endophyte isolated in our study (*Klebsiella* sp. REICA_ 034), were capable of incorporating significant amounts (between 45 and 61%) of nitrogen into the biomass of fungal gardens of *Atta cephalotes* leaf-cutter ants (Pinto-Tomás et al., 2009). Thus, considering that, both strains (REICA_142 and REICA_082) revealed the highest acetylene reduction activity, *in vitro*, amongst all tested strains, we assume that this *Enterobacter* is the major provider of biological N to cv APO plants.

In addition to fixing N, *Enterobacter* sp. REICA_082 was capable to use methanol as C source. The close assigned *Enterobacter* sp. CBMB30 and *Enterobacter arachidis* Ah-143^T were also previously identified as methylotrophic strains (Lee et al., 2006; Madhaiyan et al., 2010). Methanol is released from plant cell wall-associated pectin

polymers when demethylated during plant growth and during degradation of pectin (Kolb, 2009). Lee and colleagues (2006) showed that inoculation of rice seeds with the PGP *Enterobacter* sp. CBMB30 significant increase the concentration of CK inside rice seedlings. This phytohormone act as signal molecules and they initiate the plant cell to divide and this might leads to the demethylation of pectin and the release of methanol, which is then used by the bacterium as nutrient source for it own growth. Although this assumption has not been demonstrated, definitely the capacity to use metabolic waste products, such as methanol, might confer competitive advance over other endophytes.

The potential for beneficial effects of *Enterobacter* spp. has further been demonstrated with *Enterobacter* sp. strain 638, an endophytic inhabitant of poplar trees, which increased biomass production up to 40% when introduced into host plants that stood on marginal soils (Taghavi et al., 2009). Genomic analysis of *Enterobacter* sp. 638 revealed the presence of a genomic region that encodes the uptake and metabolism of sucrose and the synthesis of the VOCs acetoin and 2,3- butanediol (Taghavi et al. 2010). Acetoin and 2,3-butanediol are phytohormones that are involved in plant growth promotion and the induction of systemic resistance (Ryu et al. 2004). The authors suggested that the uptake of sucrose, a major photosynthate in poplar trees, by strain 638, may trigger the production of the phytohormones acetoin and 2,3-butanediol, thereby promoting plant growth. This synergistic interaction gracefully illustrates the beneficial action of particular endophytes.

Another potential N_2-fixing endophyte, *Herbaspirillum* sp. REICA_064, revealed identical *nifH* gene sequence to *nifH* of *Herbaspirillum* sp. B501 (BAD77945), a diazotrophic endophyte isolated from *Oryza officinalis* (wild rice; Elbeltagy et al., 2001). The latter strain was shown to fix N_2 in young seedlings of wild rice and in cultivated rice plants (Elbeltagy et al., 2001; Zakria et al., 2007). Further studies using this strain revealed that the *in planta nifH* transcription was enhanced in the presence of light (You et al., 2005). The process of transfer of N from the symbionts to gramineous host plants is unknown (Elmerich & Newton, 2007), however grasslands from tropical regions support the production of biomass without N amendment to the system, suggesting that N_2 fixation is common in these environments (Vitousek et al., 2002).

For all strains tested, the production of IAA was dependent on the presence of tryptophan. Tryptophan-dependent biosynthesis seems to be the main IAA biochemical pathway for plant-associated bacteria (Mehnaz et al., 2001). Moreover, IAA biosynthesis is widespread among plant-associated bacteria, which use this compound as a signalling molecule to communicate among themselves or with the host plant (Spaepen et al., 2007).

The majority of the tested strains produced relatively small amounts of IAA *in vitro*. These might be considered to represent phytostimulators (Patten & Glick, 2002), although the beneficial effects of IAA are not only related to the amount produced by the bacteria but rather to the sensitivity of the plant tissue (Spaepen et al., 2007). Large amounts of IAA produced by bacteria, together with endogenously produced plant IAA, might lead to unbalance plant growth and activation of ACC synthase to produce ACC, the immediate precursor of ET (Chen et al., 2005). ET is implicated in many physiological processes throughout the life cycle of the plant, including fruit ripening, root development and regulation of plant responses to abiotic and biotic factors. Thus, bacteria harbouring ACC deaminase (i.e. the enzyme that converts ACC to ammonia and α-ketobutyrate) might also modulate plant ET levels and consequently ameliorate plant stresses (Glick et al., 1998). It is interesting that two of three major IAA producers *in vitro* were also able to use ACC as their main N source. It is tempting to speculate that the high amount of IAA produced by these bacteria (REICA_203 and REICA_035) induces a phytohormone imbalance in the host and further production of ACC which is then used by the bacteria as N source (**Chapter 2**; Glick et al., 1998; Hardoim et al., 2008). This assumption was further supported by Toklikishvili and colleagues (2010), who observed a significant reduction in crown gall formation on tomato plants infected with either *Rhizobium tumefaciens* or *Rhizobium vitis* (both formerly *Agrobacterium*) when plants were pre-inoculated with ACC deaminase-containing bacteria. Furthermore, beneficial effects of the introduction of ACC deaminase-contained bacteria, resulting in an enhancement of the host biomass, have been reported in experiments under greenhouse conditions (Onofre-Lemus et al., 2009), albeit mainly under abiotic stress conditions such those induced by salt (Cheng et al., 2007), heavy metals (Safronova et al., 2006), phytopathogens (Wang et al., 2000), flooding (Grichko & Glick, 2001) or drought (Belimov et al., 2009).

Half or more of the selected strains showed phosphate solubilisation activity and/or siderophore production. Genes encoding proteins involved in siderophore biosysnthesis and receptors were overrepresented in the rice endophyte metagenome when compared to a selected range of metagenomes (**Chapter 7**). As most endophytes originate from the surrounding soil (Hallmann et al., 1997), we assume that the plant selects potentially beneficial bacteria and, once colonizing the rhizosphere, these might also become endophytic. In addition, siderophore-producing endophytes might outcompete phytopatogens for ferrous iron inside host plants as occurs in the rhizosphere (van der Lelie et al., 2009), thus antagonizing detrimental species and improving plant growth. The

capacity of bacteria to solubilise inorganic phosphate is often related to the secretion of organic acids. In *Enterobacter intermedium,* the production of 2-ketogluconic acid, which is derived from the oxidation of glucose, revealed a strong correlation with the solubilisation of mineral phosphate (Hwangbo et al., 2003). Other organic acids, such as acetic, citric, gluconic, glycolic, isobutyric, isovaleric, lactic, malonic, oxalic, propionic and succinic acids, have been identified among phosphate-solubilising bacteria (Hayat et al., 2010).

All these bacterial traits are important determinants of colonization efficiency. For instance, motility driven by chemotaxis confers a competitive advance to *Azospirillum brasilense* in the colonization of wheat roots (Vande Broek et al., 1998). Furthermore, the proportion of isolated strains with flagellar motility gradually increased from the rhizosphere to the endosphere of wheat roots (Czaban et al., 2007). Swimming and swarming motilities are flagellum-dependent types of cell locomotion that occur in liquid or on surface, respectively (Harshey, 2003). As endophytes often come from the root-surrounding soil, the ability to swim towards roots and to swarm up to entry sites (e.g. emergence of lateral roots, root tips, wounds) might confer significant advantages over naïve strains. In anaerobic respiration, nitrate is a prevalent terminal electron acceptor. Hence, endophytes with nitrate reduction capacity might have more niche opportunities than those without. This assumption was evaluated by Mirleau and colleagues (2001), who showed that a mutant of *Pseudomonas fluorescens* C7R12 defective in nitrate reductase was outcompeted by the wild-type strain in the rhizosphere of tomato plants.

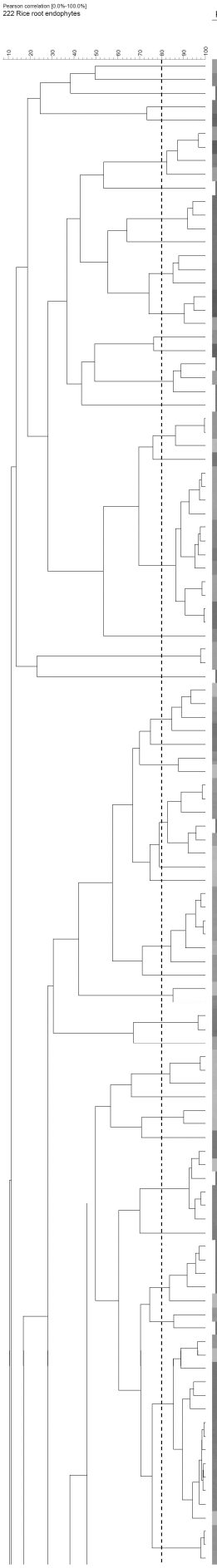
Interestingly, endophytes that were closely related regarding the 16S rRNA gene sequence revealed dissimilar metabolic profiles. For instance, *Enterobacter* sp. strain REICA_112 showed a C utilization capacity which was different from that of the other strains of this genus. Two other strains, REICA_149 and REICA_277, which were closely related to the peanut nodule - isolated alphaproteobacterium CCBAU 45397 (99.1% sequence similarity) also showed distinct metabolic profiles. These results are in agreement with the "flexible genome – lifestyle" paradigm which is apparent in bacteria such as *Escherichia coli*, where diverse strains within the species harbour a relatively conserved core genome next to a flexible (accessory) genome, which harbours a suite of adaptive genes which are selected by (incidental) evolutionary forces (van Elsas et al., 2010).

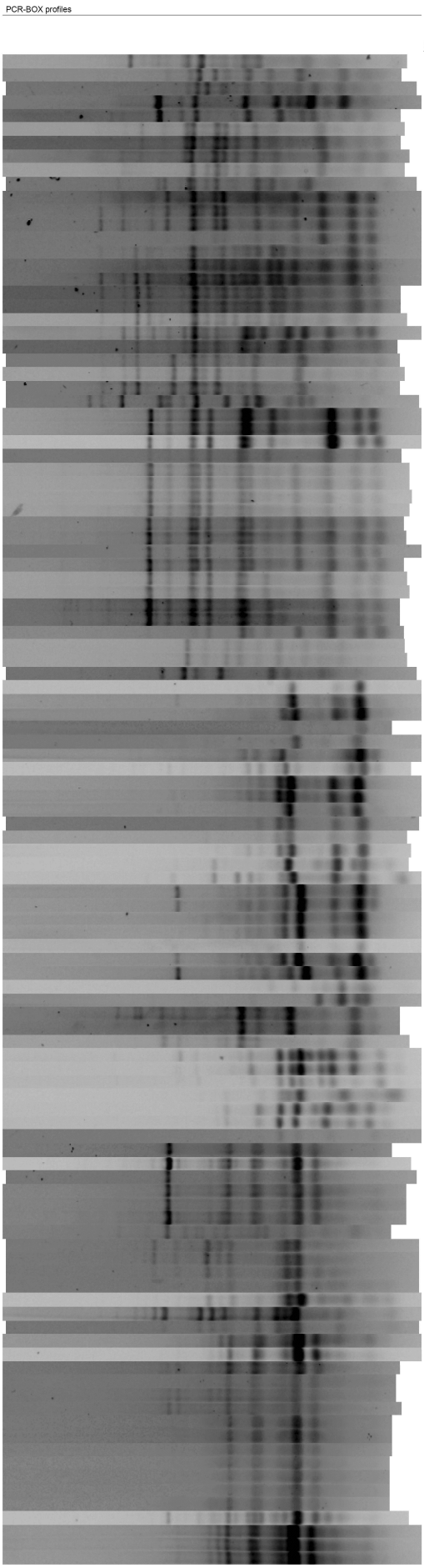
In summary, our observations suggest that the high richness of endophytes in our rice plants might have been spurred by two factors, i.e. (1) niche differentiation, which may be related to plant tissue segmentation, and (2) resource partitioning, e.g. the complementary

use of carbohydrates. This reasoning is similar to that exposed for organisms in the rhizosphere (Lugtenberg & Kamilova, 2009). Although a particular bacterial species might predominate in the system, in the present study a particular *Enterobacter* sp. which might positively affect functioning of the system (in this case, plant growth), it is only the combination of bacterial traits, shaped by evolutionary factors, and host phenotype that will set the equilibrium for better performance and sustainability.

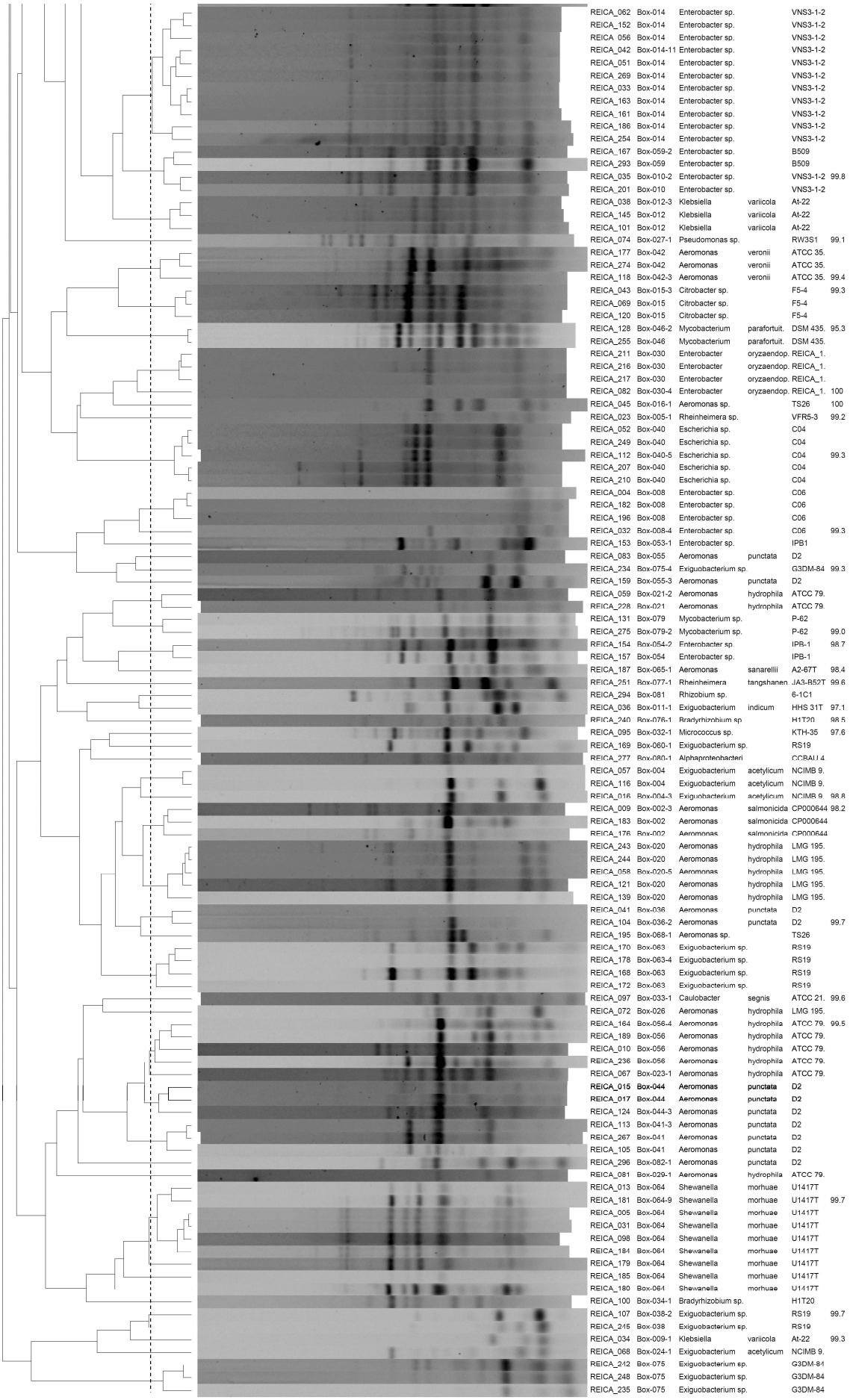
Fig. 14 Genomic fingerprinting profiles of rice endophyte isolates

Dendrogram obtained by unweighted pair group average linkage (UPGMA) after Pearson correlation analysis between BOX-PCR fingerprint profiles of 222 rice endophytes isolated from root tissues. The cut-off level of 80% was used to discriminate BOX-PCR patterns. For each BOX-PCR pattern, a representative strain was identified by sequencing the 16S rRNA gene. The closest match bacteria, strain and sequence similarity (%) are shown for representative strains (n=82).





Strain ID	BOX ID	Closest match		Strain	Sequence similarity %
REICA_175	Box-062-1	Pseudomonas sp.		22-34	100
REICA_230	Box-074-1	Enterobacter sp.		VNS3-1-2	
REICA_079	Box-028-1	Sphingomonas	pituitosa	EDIV	98.7
REICA_014	Box-003-2	Enterobacter sp.		IPB1	99.5
REICA_223	Box-003	Enterobacter sp.		IPB1	
REICA_114	Box-043	Enterobacter sp.		2B5C	
REICA_123	Box-043-4	Enterobacter sp.		2B5C	99.4
REICA_264	Box-043	Enterobacter sp.		2B5C	
REICA_262	Box-043	Enterobacter sp.		2B5C	
REICA_064	Box-022-1	Herbaspirillum sp.		B501	99.9
REICA_039	Box-013-4	Aeromonas	hydrophila	LMG 195.	99.1
REICA_162	Box-013	Aeromonas	hydrophila	LMG 195.	
REICA_202	Box-013	Aeromonas	hydrophila	LMG 195.	
REICA_060	Box-013	Aeromonas	hydrophila	LMG 195.	
REICA_096	Box-050	Aeromonas	hydrophila	LMG 195.	
REICA_137	Box-050	Aeromonas	hydrophila	LMG 195.	
REICA_144	Box-050-6	Aeromonas	hydrophila	LMG 195.	99.5
REICA_011	Box-050	Aeromonas	hydrophila	LMG 195.	
REICA_093	Box-050	Aeromonas	hydrophila	LMG 195.	
REICA_141	Box-050	Aeromonas	hydrophila	LMG 195.	
REICA_200	Box-070-1	Aeromonas	punctata	D2	
REICA_047	Box-017-1	Aeromonas	hydrophila	SBK1	98.0
REICA_003	Box-001-3	Enterobacter sp.		DHM-1T	100
REICA_265	Box-001	Enterobacter sp.		DHM-1T	
REICA_006	Box-001	Enterobacter sp.		DHM-1T	
REICA_106	Box-037	Aeromonas	hydrophila	LMG 195.	
REICA_258	Box-078-4	Enterobacter sp.		IPB1	99.3
REICA_259	Box-078	Enterobacter sp.		IPB1	
REICA_156	Box-078	Enterobacter sp.		IPB1	
REICA_155	Box-078	Enterobacter sp.		IPB1	
REICA_075	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_077	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_065	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_130	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_206	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_272	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_140	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_027	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_028	Box-006-13	Enterobacter	oryzaphylus	REICA_1.	
REICA_111	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_007	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_054	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_222	Box-006	Enterobacter	oryzaphylus	REICA_1.	
REICA_099	Box-058	Pseudomonas	putida	BIRD-1	
REICA_166	Box-058-2	Pseudomonas	putida	BIRD-1	99.8
REICA_149	Box-052-1	Alphaproteobacteri.		CCBAU 4.	99
REICA_232	Box-073	Enterobacter sp.		VNS3-1-2	
REICA_256	Box-073	Enterobacter sp.		VNS3-1-2	
REICA_224	Box-073-4	Enterobacter sp.		VNS3-1-2	
REICA_102	Box-073	Enterobacter sp.		VNS3-1-2	
REICA_125	Box-045-1	Enterobacter sp.		BF1-8	99.7
REICA_165	Box-057-2	Rheinheimera sp.		VFR5-3	
REICA_229	Box-057	Rheinheimera sp.		VFR5-3	
REICA_136	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_158	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_199	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_193	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_194	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_205	Box-035	Micrococcus sp.		BQN1N-0.	
REICA_103	Box-035-7	Micrococcus sp.		BQN1N-0.	99.9
REICA_198	Box-069-1	Rheinheimera sp.		VFR5-3	
REICA_085	Box-031-7	Rheinheimera sp.		VFR5-3	98.3
REICA_092	Box-031	Rheinheimera sp.		VFR5-3	
REICA_080	Box-031	Rheinheimera sp.		VFR5-3	
REICA_233	Box-031	Rheinheimera sp.		VFR5-3	
REICA_126	Box-031	Rheinheimera sp.		VFR5-3	
REICA_227	Box-031	Rheinheimera sp.		VFR5-3	
REICA_133	Box-031	Rheinheimera sp.		VFR5-3	
REICA_071	Box-025-2	Aeromonas	hydrophila	SBK1	
REICA_250	Box-025	Aeromonas	hydrophila	SBK1	
REICA_048	Box-018-2	Enterobacter sp.		B509	99.5
REICA_050	Box-018	Enterobacter sp.		B509	
REICA_109	Box-039-1	Rheinheimera sp.		VFR5-3	98.3
REICA_203	Box-071-3	Rhizobium sp.		6-1C1	97.7
REICA_204	Box-071	Rhizobium sp.		6-1C1	
REICA_226	Box-071	Rhizobium sp.		6-1C1	
REICA_219	Box-072-1	Bradyrhizobium sp.		H1T20	
REICA_138	Box-048-2	Aeromonas sp.		TS26	
REICA_231	Box-040	Aeromonas sp.		TS26	
REICA_192	Box-067-1	Exiguobacterium sp.		RS19	
REICA_191	Box-066-6	Enterobacter	oryzaphylus	REICA_1.	
REICA_279	Box-066	Enterobacter	oryzaphylus	REICA_1.	
REICA_132	Box-066	Enterobacter	oryzaphylus	REICA_1.	
REICA_053	Box-066	Enterobacter	oryzaphylus	REICA_1.	
REICA_214	Box-066	Enterobacter	oryzaphylus	REICA_1.	
REICA_247	Box-066	Enterobacter	oryzaphylus	REICA_1.	
REICA_029	Box-007-1	Enterobacter sp.		VNS3-1-2	
REICA_055	Box-019-5	Bradyrhizobium	liaoningense	2281T	98.9
REICA_246	Box-019	Bradyrhizobium	liaoningense	2281T	
REICA_143	Box-019	Bradyrhizobium	liaoningense	2281T	
REICA_117	Box-019	Bradyrhizobium	liaoningense	2281T	
REICA_292	Box-019	Bradyrhizobium	liaoningense	2281T	
REICA_171	Box-061-2	Bradyrhizobium sp.		H1T20	99.3
REICA_225	Box-061	Bradyrhizobium sp.		H1T20	
REICA_135	Box-047-3	Sphingomonas	pituitosa	EDIV	
REICA_208	Box-047	Sphingomonas	pituitosa	EDIV	
REICA_066	Box-047	Sphingomonas	pituitosa	EDIV	
REICA_087	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_142	Box-049-11	Enterobacter	oryzaphylus	REICA_1.	100
REICA_268	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_076	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_084	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_190	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_147	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_188	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_150	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_270	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_108	Box-049	Enterobacter	oryzaphylus	REICA_1.	
REICA_146	Box-051-3	Enterobacter sp.		2B5C	
REICA_278	Box-051	Enterobacter sp.		2B5C	
REICA_129	Box-051	Enterobacter sp.		2B5C	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REICA_056 Box-014 Enterobacter sp. VNS3-1-2
REICA_042 Box-014-11 Enterobacter sp. VNS3-1-2
REICA_051 Box-014 Enterobacter sp. VNS3-1-2
REICA_269 Box-014 Enterobacter sp. VNS3-1-2
REICA_033 Box-014 Enterobacter sp. VNS3-1-2
REICA_163 Box-014 Enterobacter sp. VNS3-1-2
REICA_161 Box-014 Enterobacter sp. VNS3-1-2
REICA_186 Box-014 Enterobacter sp. VNS3-1-2
REICA_254 Box-014 Enterobacter sp. VNS3-1-2
REICA_167 Box-059-2 Enterobacter sp. B509
REICA_293 Box-059 Enterobacter sp. B509
REICA_035 Box-010-2 Enterobacter sp. VNS3-1-2 99.8
REICA_201 Box-010 Enterobacter sp. VNS3-1-2
REICA_038 Box-012-3 Klebsiella variicola At-22
REICA_145 Box-012 Klebsiella variicola At-22
REICA_101 Box-012 Klebsiella variicola At-22
REICA_074 Box-027-1 Pseudomonas sp. RW3S1 99.1
REICA_177 Box-042 Aeromonas veronii ATCC 35.
REICA_274 Box-042 Aeromonas veronii ATCC 35.
REICA_118 Box-042-3 Aeromonas veronii ATCC 35. 99.4
REICA_043 Box-015-3 Citrobacter sp. F5-4 99.3
REICA_069 Box-015 Citrobacter sp. F5-4
REICA_120 Box-015 Citrobacter sp. F5-4
REICA_128 Box-046-2 Mycobacterium parafortuit. DSM 435. 95.3
REICA_255 Box-046 Mycobacterium parafortuit. DSM 435.
REICA_211 Box-030 Enterobacter oryzaendop. REICA_1.
REICA_216 Box-030 Enterobacter oryzaendop. REICA_1.
REICA_217 Box-030 Enterobacter oryzaendop. REICA_1.
REICA_082 Box-030-4 Enterobacter oryzaendop. REICA_1. 100
REICA_045 Box-016-1 Aeromonas sp. TS26 100
REICA_023 Box-005-1 Rheinheimera sp. VFR5-3 99.2
REICA_052 Box-040 Escherichia sp. C04
REICA_249 Box-040 Escherichia sp. C04
REICA_112 Box-040-5 Escherichia sp. C04 99.3
REICA_207 Box-040 Escherichia sp. C04
REICA_210 Box-040 Escherichia sp. C04
REICA_004 Box-008 Enterobacter sp. C06
REICA_182 Box-008 Enterobacter sp. C06
REICA_196 Box-008 Enterobacter sp. C06
REICA_032 Box-008-4 Enterobacter sp. C06 99.3
REICA_153 Box-053-1 Enterobacter sp. IPB1
REICA_083 Box-055 Aeromonas punctata D2
REICA_234 Box-075-4 Exiguobacterium sp. G3DM-84 99.3
REICA_159 Box-055-3 Aeromonas punctata D2
REICA_059 Box-021-2 Aeromonas hydrophila ATCC 79.
REICA_228 Box-021 Aeromonas hydrophila ATCC 79.
REICA_131 Box-079 Mycobacterium sp. P-62
REICA_275 Box-079-2 Mycobacterium sp. P-62 99.0
REICA_154 Box-054-2 Enterobacter sp. IPB-1 98.7
REICA_157 Box-054 Enterobacter sp. IPB-1
REICA_187 Box-065-1 Aeromonas sanarellii A2-67T 98.4
REICA_251 Box-077-1 Rheinheimera tangshanen. JA3-B52T 99.6
REICA_294 Box-081 Rhizobium sp. 6-1C1
REICA_036 Box-011-1 Exiguobacterium indicum HHS 31T 97.1
REICA_240 Box-076-1 Bradyrhizobium sp. H1T20 98.5
REICA_095 Box-032-1 Micrococcus sp. KTH-35 97.6
REICA_169 Box-060-1 Exiguobacterium sp. RS19
REICA_277 Box-080-1 Alphaproteobacteri CCBAU 4
REICA_057 Box-004 Exiguobacterium acetylicum NCIMB 9.
REICA_116 Box-004 Exiguobacterium acetylicum NCIMB 9.
REICA_016 Box-004-3 Exiguobacterium acetylicum NCIMB 9. 98.8
REICA_009 Box-002-3 Aeromonas salmonicida CP000644 98.2
REICA_183 Box-002 Aeromonas salmonicida CP000644
REICA_176 Box-002 Aeromonas salmonicida CP000644
REICA_243 Box-020 Aeromonas hydrophila LMG 195.
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REICA_058 Box-020-5 Aeromonas hydrophila LMG 195.
REICA_121 Box-020 Aeromonas hydrophila LMG 195.
REICA_139 Box-020 Aeromonas hydrophila LMG 195.
REICA_041 Box-036 Aeromonas punctata D2
REICA_104 Box-036-2 Aeromonas punctata D2 99.7
REICA_195 Box-068-1 Aeromonas sp. TS26
REICA_170 Box-063 Exiguobacterium sp. RS19
REICA_178 Box-063-4 Exiguobacterium sp. RS19
REICA_168 Box-063 Exiguobacterium sp. RS19
REICA_172 Box-063 Exiguobacterium sp. RS19
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REICA_072 Box-026 Aeromonas hydrophila LMG 195.
REICA_164 Box-056-4 Aeromonas hydrophila ATCC 79. 99.5
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REICA_010 Box-056 Aeromonas hydrophila ATCC 79.
REICA_236 Box-056 Aeromonas hydrophila ATCC 79.
REICA_067 Box-023-1 Aeromonas hydrophila ATCC 79.
REICA_015 Box-044 Aeromonas punctata D2
REICA_017 Box-044 Aeromonas punctata D2
REICA_124 Box-044-3 Aeromonas punctata D2
REICA_113 Box-041-3 Aeromonas punctata D2
REICA_267 Box-041 Aeromonas punctata D2
REICA_105 Box-041 Aeromonas punctata D2
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REICA_107 Box-038-2 Exiguobacterium sp. RS19 99.7
REICA_245 Box-038 Exiguobacterium sp. RS19
REICA_034 Box-009-1 Klebsiella variicola At-22 99.3
REICA_068 Box-024-1 Exiguobacterium acetylicum NCIMB 9.
REICA_242 Box-075 Exiguobacterium sp. G3DM-84
REICA_248 Box-075 Exiguobacterium sp. G3DM-84
REICA_235 Box-075 Exiguobacterium sp. G3DM-84

Table 4 Plant growth-promotion and adaptation traits of selected rice endophytes (n=20)

Phylum	Class	Strains	Accession number – Closest match (similarity %)	N2 fixation		IAA [b] production	Phosphatase activity	ACC deaminase	Siderophore	Oxidase	Catalase	Methylotrophic		Denitrification	Amylase	Portease [c]	Cellulase	Swimming	Swarming
				PCR product	ARA[a]							PCR product	Plate growth						
Proteobacteria	Gamma	REICA_142	AY683044 *Enterobacter* sp. CBMB30 (98.6%)	+	4,6± 0,01	-	+	+	-	+	+	-	-	+	-	-	+	+	+
		REICA_082	FJ205683 *Enterobacter* sp. C06 (99.4%)	+	0,24	13,7±2,3	+	+	-	+	+	+	+	+	-	-	+	+	++
		REICA_035	Z96079 *Enterobacter cloacae* subsp. *dissolvens* LMG 2683^T (99.7%)	-	-	59,4±3,0	-	++	-	+	+	-	-	+	-	-	-/+	+	+
		REICA_112	DQ855282 *Enterobacter* sp. FMB1 (99.5%)	-	-	-	+	+	-	+	+	-	-	+	-	-	+	+	+
		REICA_043	EF491837 *Citrobacter* sp. F5-4 (98.9%)	-	-	15,1±4,9	+	++	-	+	-	-	-	+	-	-	-	+	nd
		REICA_034	CP001819 *Klebsiella variicola* At-22 (99.3%)	+	0,05	-	+	+	-	+	+	-	-	+	-	-	+	+	++
		REICA_164	X60404 *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966^T (99.8%)	-	-	14,7±1,9	-	+	+	+	-	-	-	+	+	7,8± 0,8	-	+	++
		REICA_181	FJ589031 *Shewanella xiamenensis* S4^T (99.9%)	-	-	2,9±2,0	-	+	+	+	+	-	-	+	-	2,7± 0,7	-	+	++
		REICA_175	EU167979 *Pseudomonas* sp. 22-34 (100%)	-	-	-	-	++	+	+	+	+	+	++	-	-	-	+	++
	Beta	REICA_064	AB049133 *Herbaspirillum* sp. B501 (99.9%)	+	0,03	-	-	++	+	-	+	+	-	+	-	-	-	-	+
	Alpha	REICA_055	AF363132 *Bradyrhizobium liaoningense* 2281^T (98.9%)	-	-	-	+	-	+	-	-	-	-	+	-	-	-	+	-
		REICA_240	AF363132 *Bradyrhizobium liaoningense* 2281^T (98.5%)	-	-	-	+	-	+	+	-	-	-	-/+	-	-	-	+	nd
		REICA_149	HM107183 *Alphaproteobacterium* CCBAU 45397 (98.7%)	-	-	-	+	nd	-	-	nd	-	-	+	-	-	-	-	-
		REICA_277	HM107183 *Alphaproteobacterium* CCBAU 45397 (99.0%)	-	-	-	+	-	+	-	nd	-	-	+	-	-	-	-	-
		REICA_203	EU142838 *Rhizobium* sp. J3-N19 (98.3%)	-	nd	86,5±0,6	nd	+	+	-/+	+	-	-	nd	nd	nd	+	nd	-
		REICA_079	NR_025363 *Sphingomonas pituitosa* EDIV^T (98.7%)	-	-	0,4±0,7	-	++	+	-/+	+	-	-	-/+	-	-	-	-	-
		REICA_097	CP002008 *Caulobacter segnis* ATCC 21756^T (99.6%)	-	-	4,4±2,4	-	-	+	+	-	-	-	-	-	-	-/+	-	-
	Bacilli	REICA_016	DQ019167 *Exiguobacterium acetylicum* DSM 20416^T (98.8%)	-	-	-	+	+	-	+	+	-	-	-	+	1,3± 0,4	-	+	+
Actino bacteria		REICA_095	HM854237 *Micrococcus* sp. KTH-35 (97.6%)	-	-	48,8±2,9	-	-	+	-	+	-	+	-	-	-	-	-	-
		REICA_128	NR_026285 *Mycobacterium parafortuitum* DSM 43528^T (95.3%)	-	-	-	-	+	+	-	+	-	-	-	-	-	-/+	-	-

^{T} quantification of activity are: absent (-); weak activity (-/+); activity (+); high activity (++); non determined (nd)

[a] % of acetylene reduced $24h^{-1}$ $tube^{-1}$

[b] µg ml^{-1} of IAA produced

[c] mm of clearing zones

Chapter 5

Enterobacter oryziphilus sp. nov. and *Enterobacter oryzendophyticus* sp. nov., isolated from the endosphere of rice roots

Pablo R. Hardoim, Rashid Nazir, Angela Sessitsch, Leo S. van Overbeek, Jan D. van Elsas[1]

Abstract

Six Gram-negative, facultatively anaerobic, non-spore-forming, nitrogen-fixing, rod-shaped isolates were obtained from the root endosphere of rice grown at the IRRI and investigated in a polyphasic taxonomic study. Comparative 16S rRNA and *rpoB* gene sequence analyses allocated the isolates within the family *Enterobacteriaceae,* with *Enterobacter radicincitans, E. arachidis* and *E. cowanii* as the closest relatives. On the basis of the phylogenetic analyses, DNA–DNA hybridization data and unique biochemical characteristics, the isolates were shown to belong to the genus of *Enterobacter,* and were distinguishable into distinct groups that represent two novel species. These two species can be differentiated from each other and their nearest neighbours by the following characteristics: utilization of adonitol, D-arabitol, m-inositol, L-aspartic acid, D-melibiose, D-raffinose, decarboxylation of ornithine and positive methyl red test. Both species revealed PGP activities as well as adaptation to the host plant. It is proposed that these novel isolates are classified as *Enterobacter oryziphilus* sp. nov. (type strain REICA_142^T=NCCB 100393^T) and *Enterobacter oryzendophyticus* sp. nov. (type strain REICA_082^T=NCCB 100390^T).

[1] The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *E. oryziphilus* strains REICA_084, REICA_142^T and REICA_191 are JF795012, JF795013 and JF795014, and of *E. oryzendophyticus* strains REICA_032, REICA_082^T and REICA_211 are JF795010, JF795011 and JF795015, respectively. The accession numbers for the *rpoB* gene sequences of strains strains REICA_084, REICA_142^T and REICA_191 are JF795018, JF795019 and JF795020, and of *E. oryzendophyticus* strains REICA_032, REICA_082^T and REICA_211 are JF795016, JF795017 and JF795021, respectively.

Plants interact with a great diversity of microorganisms, including enteric bacteria. These interactions, which are ruled by the characteristics of both host plant and bacteria, result in either commensalistic, mutualistic, symbiotic or parasitic relationships between both partners. For instance, *Enterobacter cloacae* subsp. *dissolvens*, previously described as *Erwinia dissolvens*, was first isolated from diseased corn (Brenner *et al.,* 1986), whereas it was also found in the endosphere of rice plants without causing harm to the host (Prakamhang *et al.,* 2009). Within the genus *Enterobacter,* many other species have been reported to interact intimately with their host plant. For instance, *E. cancerogenus* NCPPB 2176^T, *E. nimipressuralis* ATCC 9912^T and *E. pyrinus* ATCC 49851^T were isolated, respectively, from poplar, elm and pear trees, in which they are known as phytopathogens (Dickey *et al.*, 1988; Brenner *et al.*, 1986; Chung *et al.*, 1993). On the other hand, organisms such as *E. radicincitans* D5/23^T, *E. arachidis* Ah-143^T, *Enterobacter* sp. 638, *E. oryzae* Ola-51^T and *Enterobacter* sp. CBMB30, which were isolated from respectively the phyllosphere of wheat, rhizosphere of groundnut and endosphere of poplar and rice species (i.e. *Oryza latifolia* and *O. sativa*), are known as PGPB (Madhaiyan *et al*., 2010; Taghavi *et al*., 2009; Peng *et al*., 2009; Lee *et al*., 2006).

In a recent study, we assessed the endophytic community from root tissues of rice plants by a 16S rRNA gene clone library (i.e. 530 clones were sequenced) and by cultivation (**Chapter 4**). From the clone library, ca. 30% of the sequences were assigned to one unique operational taxonomic unit (OTU), at 99% sequence similarity. In addition, we isolated a great number of bacteria (222 colonies) by serial dilution on R2A agar medium. After screening these strains for similar genotypes (*i.e.* BOX), 82 distinct fingerprinting patterns were observed, at 80% similarity cut-off level (**Chapter 4**). Analysis of the 16S rRNA genes from the culturable endophytes revealed a set of six strains that were closely related (at least 99.5% sequence similarity) to the most abundant OTU from the clone library. Phylogenetic analysis revealed that these sequences were divergent from the most closely related *Enterobacter* type strains *E. oryzae* Ola-51^T (98.9% sequence similarity), *E. radicincitans* D5/23^T (98.7%) and *E. arachidis* Ah-143^T (98.5%), but closely related to *Enterobacter* sp. CBMB30 (99.5% sequence similarity), a rice endophytic bacterium isolated from South Korea with PGP properties (Madhaiyan *et al*., 2004b). The selected six strains were further characterized in this study and, based on the results, they are proposed to represent two novel species of the genus *Enterobacter*.

Rice (*O. sativa* L.) plants cv. APO were sampled from the homogenized (rotary spading, once yearly) loamy paddy field, located at the IRRI (Los Baños, Philippines).

Roots (150 g) devoid of rhizosphere soil were surface-sterilized and endophytic bacterial cell pellets were obtained (**Chapter 7**). The bacterial cell pellets were maintained in cold thermal flasks until further isolation (*i.e.* two days). Strains REICA_142^T (=NCCB 100393^T), REICA_084 (=NCCB 100392), REICA_191 (=NCCB 100394), REICA_082^T (=NCCB 100390^T), REICA_032 (=NCCB 100389) and REICA_211 (=NCCB 100391) were isolated on R2A agar medium incubated at 28 ºC for 3 days in the presence of air. The strains were streaked to purity and cultures were stocked in 20% glycerol at -80 ºC.

Colony morphology was determined as described by Gerhardt *et al.* (1994) using Tryptone Soya Agar (TSA) as growth medium. Cellular morphology and motility were examined by phase contrast microscopy using a light microscope (Carl Zeiss, Germany). Cell dimensions were measured with a 10x ocular and 100x objective (/1.25). Motility tests were performed on R2A broth medium with 0.4% agar in accordance with Gerhardt *et al.* (1994). Gram staining was carried out with the Gram staining kit of Sigma-Aldrich (Steinheim, Germany). Cells of strains REICA_142^T, REICA_084, REICA_191, REICA_082^T, REICA_032 and REICA_211 were facultatively anaerobic, Gram-negative, motile and straight rod-shaped (0.8-1.0 x 1.8-3.0 μm). After 24 h incubation at 37 ºC on TSA, colonies were flat, translucent, regular-shaped and beige-pigmented. After an extended period of incubation, colonies of strain REICA_082^T showed filiform margins, whereas those of the other strains did not show this phenomenon.

The effects of different temperatures on growth were determined on R2A agar plates (Difco, Detroit, MI, USA) incubated at 8, 15, 23, 28, 30, 37, 42, 50 and 65 ºC. Tests for resistance to ampicillin, chloramphenicol, colistin sulphate, kanamycin, nalidixic acid, nitrofurantoin, streptomycin and tetracycline were performed using Mastring-S M26 antibiotic discs (Mast diagnostic, Bootle, UK). Tests for resistance to rifampicin (25 ug ml^{-1}) and gentamicin (25 ug ml^{-1}) were performed on R2A and LB agar media at 28 ºC. Salt tolerance was tested in a concentration range of 1, 2.5, 5, 7.5 and 10% NaCl (w/v) in R2A broth medium incubated at 37 ºC. All strains grew at temperatures between 15 and 42 ºC and in the presence of up to 5% NaCl. All strains were catalase-positive and oxidase-negative. Strains REICA_082^T and REICA_142^T are resistant to ampicillin (25 μg), nalidixic acid (30 μg) and nitrofurantoin (50 μg); however, they were sensitive to streptomycin (25 μg), rifampicin and gentamicin (25 μg ml^{-1}), kanamycin (30 μg), colistin sulphate and tetracycline (100 μg). Strain REICA_142^T was also resistant to chloramphenicol (50 μg). On the other hand, strain REICA_082^T was not.

Utilization of carbonaceous compounds was determined using the 95 compounds contained in Biolog GN2 microplates (Hayward, CA, USA) after an incubation period of 48h at 28 ºC. The six strains could be separated into two groups on the basis of their biochemical patterns and were differentiated by the following characteristics: strains REICA_142^T, REICA_084 and REICA_191 were negative for ornithine decarboxylase, whereas strains REICA_082^T, REICA_032 and REICA_211 were positive. Moreover, the latter group of strains could utilize the following substrates as sole carbon sources: α-cyclodextrin, glycogen, Tween 40, D-melibiose, D-raffinose, acetic acid, formic acid, *β*-hydroxybutyric acid, malonic acid, L-proline, inosine and thymidine. On the other hand, strains REICA_142^T, REICA_084 and REICA_191 utilized cis-aconitic acid, D-alanine, L-alanylglycine, L-aspartic acid and L-glutamic acid as sole carbon source (Table 5).

Several PGP properties and bacterial adaptation to host plants were investigated. The production of IAA (Gordon & Weber, 1951) and fixation of N_2 (Elbeltagy *et al*., 2001) were evaluated in test tubes after incubation at 30 and 37 °C, respectively. The production of siderophores (Schwyn & Neiland, 1987), amylases**,** cellulases and proteases, as well as the solubilization of phosphate (Nautiyal *et al*., 1999; Gerhardt *et al*., 1994) were tested on their respective medium. Furthermore, growth on so-called "copiotrophic" (C) and "oligotrophic" (O) media (Semenov *et al*., 1999), on DF (Dworking and Foster) salt with ACC as the sole nitrogen source (Penrose & Glick, 2003) and on M9 salt agar amended with 1% (v/v) methanol and 0.3% (w/v) NH_4 as sole carbon and nitrogen sources (modified from Corpe, 1985) were performed on Petri dishes after 5 days of incubation at 37 °C. The *mxaF* and *nifH* genes, encoding, respectively, the large subunit of methanol dehydrogenase and nitrogenase reductase, were subjected to PCR amplification using genomic DNA templates. Genomic DNA was extracted with the Wizard Genomic DNA purification kit (PROMEGA, Madison, USA). The genes *mxaF* and *nifH* were amplified with primers maxF-f1003 – maxF-r1561 (McDonald & Murrell, 1997) and PolF - PolR (Poly *et al.*, 2001), using the same PCR mixture composition and thermo cycling programs as described in both references. Strain REICA_082^T produced IAA (4,12 μg ml^{-1}; ±0,68) in the presence of L-tryptophan, whereas REICA_142^T did not. Both strains showed production of siderophores, ketogluconate and the solubilization of phosphate via acidification but not alkalinization. Ketogluconate is the salt compound of the organic acid 2-ketogluconic acid. This organic acid is produced by PSB and is known to be involved in the solubilization of insoluble inorganic phosphates (Hwangbo *et al*., 2003). Cellulase activity, growth on the C and O media were observed for both strains, but no amylase and

protease activities were registered. Strain REICA_082^T showed growth on M9 salt agar amended with methanol, but not REICA_142^T. In semi-solid Rennie medium (0.2% agar) strains REICA_142^T and REICA_082^T reduced, respectively, 3.66% (±0.02) and 0.24% (±0.0002) of acetylene to ET during 24h of incubation at 37 °C. No acetylene reduction was observed on bacterial cells that were inactivated after boiling the liquid culture for 10 min.

The colonization of rice plants was evaluated *in vivo* using a rifampicin resistant mutant of strain REICA_142^T, denoted REICA_142^{TR}. The mutant was selected from R2A medium amended with 25 μg ml^{-1} rifampicin (Sigma-Aldrich, St. Louis, MO). One-day-old germinated rice seeds were incubated for 1 h with 2.3 x 10^8 cells of REICA_142^{TR} CFU ml^{-1} (REICA_142^{TR} treatment) or with sterile phosphate buffered saline (PBS - pH 6.5; control treatment) (Andreote *et al*., 2009). For each treatment, four replicate rice seedlings were grown in autoclaved as well as natural V soil (Inceoglu *et al*., 2010) for a period of 4 weeks at 70% water-holding capacity (WHC). Water lost from these pots was replaced daily using sterile demineralized water. Following growth, the rice plants were surface-sterilized (Hurek *et al*., 1994), rice tissue was unlocked with mortar and pestle and serial dilutions of the resulting homogenates were made. Following plating of appropriate dilutions onto selective agar (R2A supplemented with rifampicin), and plate incubations at 28°C for 72h, the endophytic bacterial communities were thus enumerated. The ability of strain REICA_142^{TR} to invade rice plants was confirmed by isolating colonies from the relevant plates (at least 1 per replicate) and performing BOX-PCR on selected colonies (Rademaker *et al*., 2004). All inoculated rice seedlings showed invasion by strain REICA_142^{TR}. Plants growing in sterile soil were infested with 6.3 Log_{10} CFU g^{-1} fresh root tissue (±0.5) and 4.1 Log_{10} CFU g^{-1} fresh shoot tissue (±0.2), whereas plants from non-sterile soil revealed lower numbers of cells, with 4.6 Log_{10} CFU g^{-1} fresh root tissue (±0.7) and 3.6 Log_{10} CFU g^{-1} fresh shoot tissue (±0.4). No bacterial growth was observed on plates that received homogenates from uninoculated rice plants (all dilutions, leading to the conclusion that their numbers were below Log 2 per g FW). Under the experimental conditions used, no significant differences in plant FW (g) were noticed between inoculated and control plants. In sterile soil, the FW of inoculated rice seedlings was 0.83 g (±0.44), while uninoculated plants weighed 0.82 g (±0.26). Rice plants growing in non-sterile soil revealed lower FW, i.e. 0.31 g (±0.08) for inoculated plants and 0.23 g (±0.22) for uninoculated ones. The initial microbiota in the unsterilized soil thus appeared to impair the growth of rice plants in the V soil used, when compared to sterilized soil.

Sequencing of the 16S rRNA gene fragments was performed according to Weisburg *et al.* (1991). The identity of the almost-complete 16S rRNA gene sequences were determined by alignment against a curated database (Silva release 102 non-redundant reference) using the ARB package. Alignments were refined by visual inspection. The evolutionary history of 28 nucleotide sequences was inferred using the Maximum-Likelihood, Neighbor-joining and Minimum Evolution methods with MEGA5 (Tamura *et al*., 2011). The 16S rRNA gene sequences of strains REICA_084 and REICA_142^T were identical and there was 99.9% sequence similarity with REICA_191. These three strains formed a separate branch in the phylogenetic tree supported by bootstrap analysis (90%; Fig. 15). The strains clustered most closely with *E. arachidis* Ah-143^T (99.2 and 99.1%), *Enterobacter* sp. CBMB30 (99.1 and 99.0% sequence similarities), *E. oryzae* Ola-51^T (98.7 and 98.8%), *E. radicincitans* D5/23^T (98.6 and 98.5%), and with strains REICA_082^T, REICA_032 and REICA_211 (97.8 and 98.0%, respectively). The sequences of strains REICA_082^T, REICA_032 and REICA_211 were almost identical (> 99.8% sequence similarity), forming a separate branch supported by bootstrap analyses (77%, Fig. 15). The closest related species were *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^T (99.1% sequence similarity), *Enterobacter cloacae* subsp. *dissolvens* ATCC 23373^T (98.9%), *E. arachidis* Ah-143^T (98.6%) and *Enterobacter* sp. CBMB30 (98.5%). The six strains together constituted a major cluster with other members of *Enterobacter,* such as *E. cowanii* CIP 107300^T, *E. turicensis* LMG 23730^T, *E. helveticus* LMG 23732^T, *E. pulveris* LMG 601/05^T. This suggests that these strains belong to the genus *Enterobacter*.

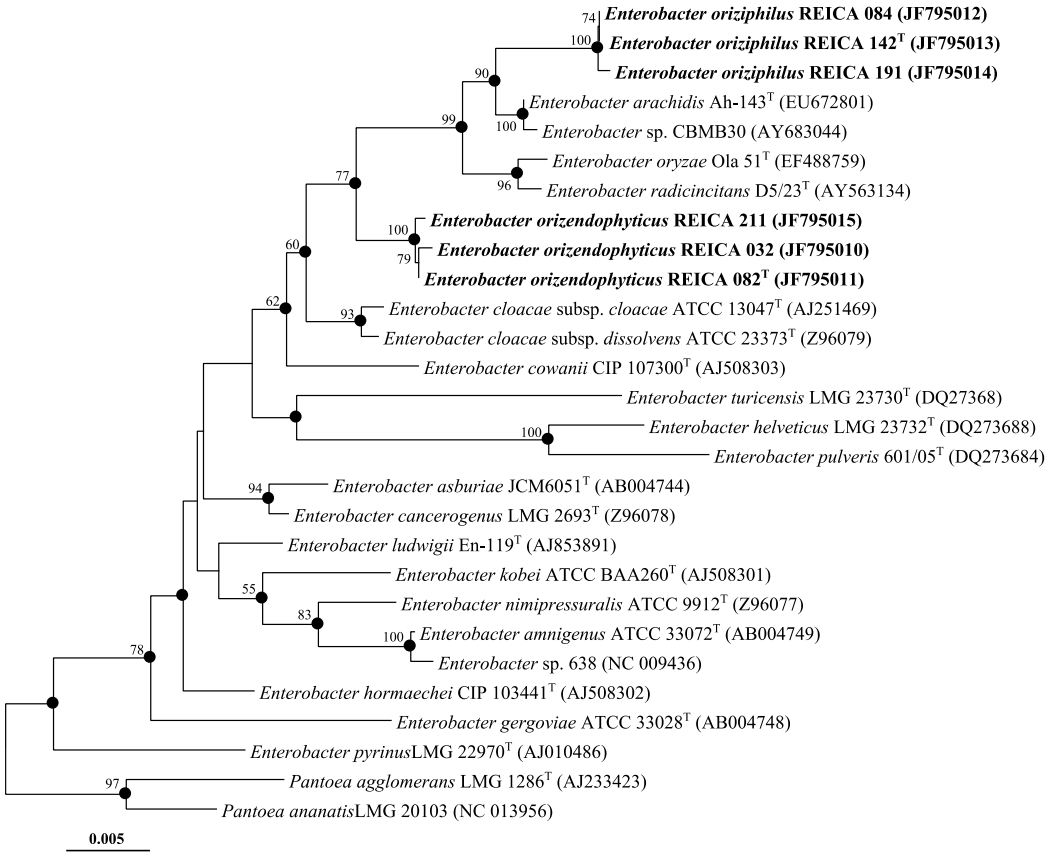

Fig. 15 Phylogenetic analysis of 16S rRNA gene from *Enterobacter* species

Minimum Evolution tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic position of *Enterobacter oryziphilus* sp. nov. and *Enterobacter oryzendophiticus* sp. nov. within the genus *Enterobacter*. A total of 28 nucleotide sequences (with 135 variable positions from a total of 1343) were used. The evolutionary distances were computed using the Maximum Composite Likelihood method. The percentage of replicates tree (> 50%) in which the associated sequence clustered together in the bootstraap test (1000 replicates) are shown next to the branches. Filled circles indicate that corresponding nodes were also recovered in trees generated with the Neighbor-Joining and Maximum-Likelihood methods. The tree is drawn to scale, with bar indicating 0.5% nucleotide substitutions. Sequences from *Pantoea* genus were used as outgroup.

The resolution of the 16S rRNA gene for identification of species within the *Enterobacteriaceae* is ambiguous in the sense that boot strap values are low within this taxonomic group (Mollet *et al*., 1997). Hence, we used a second phylogenetic marker, i.e. the *rpoB* gene, which encodes the *β*-subunit of RNA polymerase. The *rpoB* gene has higher discriminatory power than the 16S rRNA gene and is recommended for the proper allocation of new species as by Mollet *et al*. (1997). Moreover, recently sequences of the *rpoB* gene were suggested to be highly correlated with the G+C% of the bacterial genome, as well as with DNA-DNA hybridization values (Adekambi *et al*., 2009). Thus, *rpoB* has become an important candidate gene in taxonomic studies for the fine discrimination of closely related strains. The *rpoB* gene fragments were amplified and sequenced by targeting the highly variable region between positions 1300 and 2400 using primers CM_7

and CM_{31b} (Mollet *et al*., 1997). Sequence assembly was performed by using the DNA baser software (Heracle BioSoft). For identification, *rpoB* gene sequences from the six novel strains were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) against a generated *rpoB* gene database with a total of 170 sequences. These consisted of 121 sequences from complete genomes of *Enterobacteriaceae* (retrieved from Integrated Microbial Genomes (IMG) database v.3.2; http://img.jgi.doe.gov/cgi-bin/w/main.cgi), 19 sequences from type strains of *Enterobacter* species (http://www.ncbi.nlm.nih.gov/) and 30 sequences of other members of the *Enterobacteriaceae* (Mollet *et al*., 1997; Stephan *et al*., 2007). Alignments were refined by visual inspection. The evolutionary history of a selected 33 nucleotide sequences was inferred using the Maximum-Likelihood, Neighbor-joining and Minimum Evolution methods with MEGA5 (Tamura *et al*., 2011). The *rpoB* gene sequences of strains REICA_142^T, REICA_084 and REICA_191 were identical. These three strains formed a separate branch (98% bootstrap analysis) in the phylogenetic tree (Fig. 16), closely grouping with a cluster containing *E. radicincitans* D5/23^T (97.5% sequence similarity), *E. arachidis* Ah-143^T (96.7%) and *E. cowanii* CIP 107300^T (92.9%). The sequences of strains REICA_032 and REICA_211 were indistinguishable, showing 99.8% sequence similarity to that of REICA_082^T. These strains thus formed a well-defined cluster that differed from that of the other strains. This was well supported by high bootstrap values (71%, Fig. 16). The sequences of strains REICA_032 and REICA_082^T were closest related to *E. radicincitans* D5/23^T with 92.6 and 92.8% and *E. arachidis* Ah-143^T with 92.1 and 92.3% sequence similarity, respectively. The *rpoB* gene sequence of strains REICA_142^T, REICA_084 and REICA_191 on the one hand, and of strain REICA_082^T on the other hand showed 92.4% sequence similarity. As observed in the phylogenetic analysis of the 16S rRNA gene, the six strains constituted a major cluster (97% bootstrap analysis) with other members of *Enterobacter,* such as *E. cowanii* CIP 107300^T, *E. turicensis* LMG 23730^T, *E. helveticus* LMG 23732^T, *E. pulveris* LMG 601/05^T. In addition, when the *rpoB* gene sequences of all six strains were compared with those of the closest neighbours, the sequence similarity was lower than the cut-off level of 98%, which was suggested to define species within the family *Enterobacteriaceae* (Mollet *et al*., 1997). These analyses confirm that the rice endophyte strains represent different species within this genus.

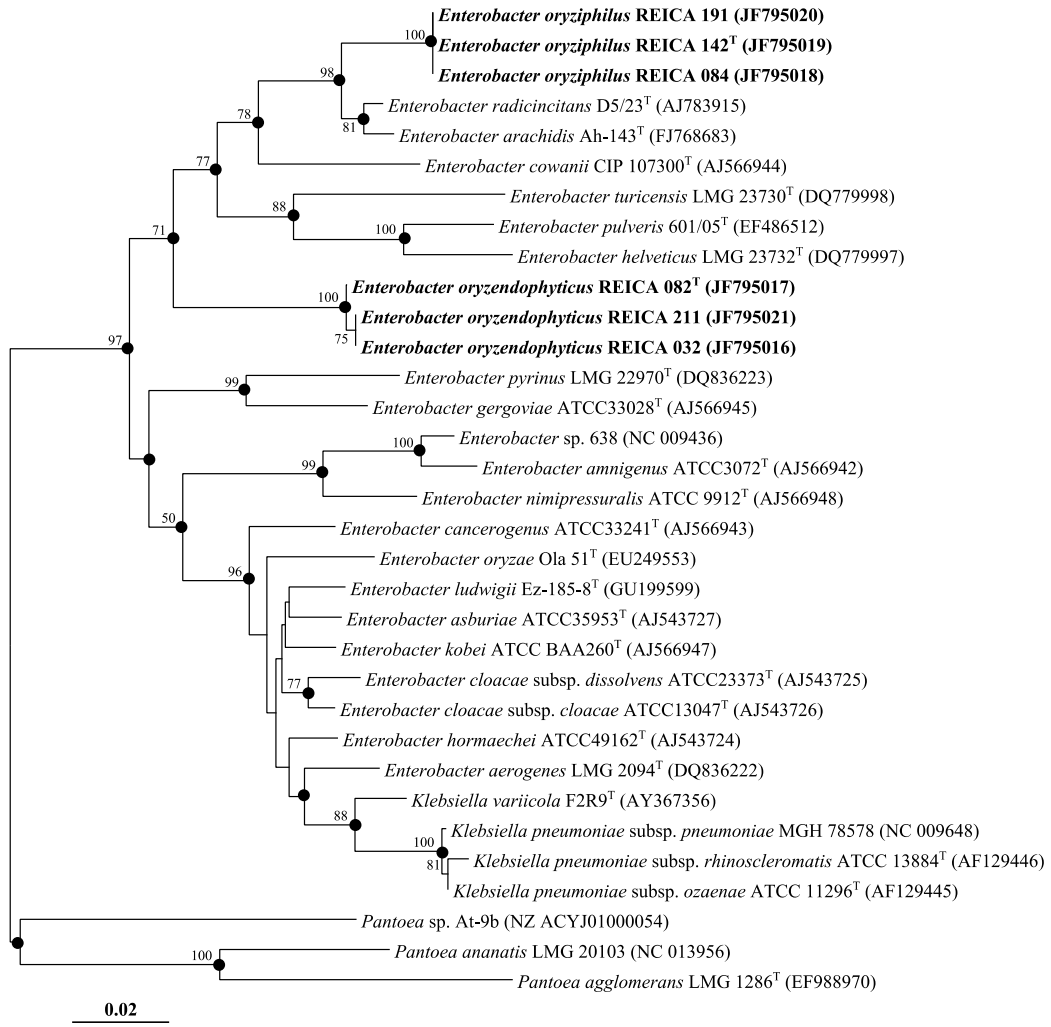

Fig. 16 Phylogenetic analysis of *rpoB* gene from *Enterobacter* species

Minimal Evolution tree based on partial *rpoB* sequences showing the phylogenetic position of *Enterobacter oryziphilus* sp. nov. and *Enterobacter oryzendophiticus* sp. nov. within the genus *Enterobacter*. A total of 33 nucleotide sequences (with 153 variable positions from a total of 495) were used. The evolutionary distances were computed using the Maximum Composite Likelihood method. The percentage of replicates tree (> 49%) in which the associated sequence clustered together in the bootstraap test (1000 replicates) are shown next to the branches. Filled circles indicate that corresponding nodes were also recovered in trees generated with the Neighbor-Joining and Maximum-Likelihood methods. The tree is drawn to scale, with bar indicating 2% nucleotide substitutions. Sequences from *Pantoea* genus were used as outgroup.

To assess whether these novel strains represent two independent genospecies within the genus *Enterobacter*, strains REICA_032, REICA_082^T, REICA_142^T, REICA_191 and their closest type strains *Enterobacter cowanii* LMG 23569^T, *Enterobacter radicincitans* LMG 23767^T, *Enterobacter oryzae* LMG 24251^T and *Enterobacter arachidis* LMG 26131^T were subjected to DNA-DNA hybridization. These analyses were carried out at the University of Gent (laboratory for microbiology) as routine procedure. Fluorometric hybridizations were achieved in the presence of 50% formamide at 45 °C, according to a modification of the method described by Ezaki *et al.* (1989). The DNA:DNA relatedness

percentages reported are the means of at least four hybridizations. The values given between brackets are the differences between the reciprocal values. The DNA:DNA hybridization results revealed strain REICA_142^T to have a DNA-DNA relatedness of 93% (±6) to REICA_191, whereas strain REICA_082^T revealed a DNA-DNA relatedness of 89% (±4) with REICA_032. The DNA-DNA relatedness between type strains of the different genospecies falls within a range of 43% (±8), with the exception of *Enterobacter oryzae* LMG 24251^T which showed 79 and 71% (±6) DNA-DNA relatedness with *Enterobacter radicincitans* LMG 23767^T and *Enterobacter arachidis* LMG 26131^T, respectively. Strain REICA_142^T showed a low level of DNA-DNA relatedness with the close relatives *E. radicincitans* D5/23^T (59%), *E. arachidis* Ah-143^T (63%) and *E. cowanii* CIP 107300^T (35%). Similar results were observed for strain REICA_082^T, which revealed low DNA-DNA relatedness with *E. radicincitans* D5/23^T (35%), *E. arachidis* Ah-143^T (31%), *Enterobacter oryzae* LMG 24251^T (41%) and *E. cowanii* CIP 107300^T (33%).

The overall DNA G+C content was determined according to the HPLC method (Mesbah et al., 1989) using the DNA prepared for DNA–DNA hybridizations. The values (means of three independent analyses of the same DNA sample) for REICA_032, REICA_082^T, REICA_142^T and REICA_191 were 52.7, 52.9, 52.1 and 51.7 mol%, respectively. These values are consistent with the DNA G+C contents of other members of the genus *Enterobacter* (Madhaiyan *et al*., 2010).

Description of *Enterobacter oryziphilus* sp. nov.

Enterobacter oryziphilus (o.ry`za. L. nom. n. *oryza*, rice; *philus* gr. masc. adj. *philos*, friend, loving; N.L. masc. adj. oryzaphilus, a rice lover).

Cells are Gram-negative, motile, straight rods (0.9-1.0 μm wide by 1.8-2.9 μm long) and occur singly or in pairs. Mesophilic, chemoorganotrophic and aerobic to facultatively anaerobic. Colonies on TSA medium are beige pigmented, 2-3 mm in diameter and convex after 24 h at 37°C. Growth occurs at 15-42°C (optimum 28-37°C). NaCl inhibits growth at concentrations above 5%. Growth was detected on C and O media. Cytochrome oxidase negative and catalase positive. The type strain is resistant to ampicillin (25 μg), nalidixic acid (30 μg), chloramphenicol and nitrofurantoin (50 μg), and sensitive to streptomycin (25 μg), rifampicin and gentamicin (25 μg ml^{-1}), kanamycin (30 μg), colistin sulphate and tetracycline (100 μg). Showed a positive reaction for Voges–Proskauer, arginine

dihydrolase, malonate decarboxylase, citrate utilization, esculin hydrolysis and methyl red test; revealed to be negative for urease, gelatin hydrolysis, hydrogen sulfide and indole production, lysine and ornithine decarboxylase. Nitrate and acetylene reduction, phosphate solubilisation and cellulase are positive, whereas amylase, protease and production of IAA are negative. Positive for utilization of adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, *α*-D-glucose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, *β*-methyl-D-glucoside, D-psicose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl-ester, cis-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, D,L-lactic acid, D-saccharic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, glycerol, D,L-*α*-glycerol phosphate, *α*-D-glucose-1-phosphate, D-glucose-6-phosphate, dextrin, Tween 80, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine. The following compounds are not utilized as sole carbon source: *i*-erythritol, D-melibiose, D-raffinose, acetic acid, formic acid, D-galactonic acid lactone, *α*-hydroxybutyric acid, *β*-hydroxybutyric acid, *γ*-hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, *α*-keto butyric acid, *α*-keto glutaric acid, *α*-keto valeric acid, malonic acid, propionic acid, quinic acid, sebacic acid, succinamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-threonine, D,L-carnitine, *γ*-amino butyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, *α*-cyclodextrin, glycogen and Tween 40. The *nifH* gene for nitrogenase activities was detected in the genomic DNA, but not *mxaF* gene for methanol dehydrogenase for strains REICA_142^T, REICA_084 and REICA_191. The DNA G+C contents of strains REICA_142^T and REICA_191 are 52.1 and 51.7 mol%, respectively. The 16S rRNA and *rpoB* gene sequences are deposited at the GenBank data bank under the accession numbers JF795013 and JF795019 for REICA_142^T, respectively.

The type strain, REICA_142^T (=NCCB 100393^T), was isolated from internal root tissues of rice (*Oryza sativa* L.) cultivar APO. The samples were collected at flowering stage from an experimental paddy field at the IRRI, Philippines.

Description of Enterobacter oryzendophyticus sp. nov.

Enterobacter oryzendophyticus (o.ry`za. L. gen. n. *oryzae*, of rice; Gr. pref. *endo*-, within; Gr. neutr. n. *phyton*, plant; L. masc. suff. -*icus*, suffix used with the sense of pertaining to; N.L. masc. adj. oryzaendophyticus, within rice plant, pertaining to the original isolation from rice tissues).

Cells are Gram-negative, motile, straight rods (0.8-1.0 μm wide by 1.8-3.0 μm long) and occur singly or in pairs. Mesophilic, methylotrophic, chemoorganotrophic and aerobic to facultatively anaerobic. Colonies on TSA medium are beige pigmented, 1-1.5 mm in diameter and convex after 24 h at 37°C. Growth occurs at 15-42°C (optimum 28-37°C). NaCl inhibits growth at concentrations above 5%. Growth was detected on C and O media and on M9 salt amended with 1% (v/v) methanol as sole carbon source. Cytochrome oxidase negative and catalase positive. Resistant to ampicillin (25 μg), nalidixic acid (30 μg) and nitrofurantoin (50 μg), however, sensitive to streptomycin (25 μg), rifampicin and gentamicin (25 μg ml^{-1}), kanamycin (30 μg), chloramphenicol (50 μg), colistin sulphate and tetracycline (100 μg). Showed a positive reaction for Voges–Proskauer, arginine dihydrolase, malonate and ornithine decarboxylase, citrate utilization, esculin hydrolysis and methyl red test; negative for urease, gelatin hydrolysis, hydrogen sulfide and indole production and lysine decarboxylase. Nitrate and acetylene reduction, phosphate solubilization, cellulase and production of IAA were positive, while amylase and protease were negative. Positive for utilization of adonitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, *α*-D-glucose, m-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, *β*-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl-ester, acetic acid, bromosuccinic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, *β*-hydroxybutyric acid, D,L-lactic acid, malonic acid, D-saccharic acid, succinic acid, glucuronamide, L-alaninamide, L-alanine, L-asparagine, L-histidine, L-proline, L-serine, inosine, thymidine, glycerol, D,L-*α*-glycerol phosphate, *α*-D-glucose-1-phosphate, D-glucose-6-phosphate, dextrin, *α*-cyclodextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine. The following compounds were not utilized as sole carbon source: *i*-erythritol, *α*-hydroxybutyric acid, *α*-keto butyric acid, *α*-keto glutaric acid, *α*-keto valeric acid, quinic acid, cis-aconitic acid, itaconic acid, propionic acid, sebacic acid, succinamic acid, L-pyroglutamic acid, L-aspartic acid, L-

glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, *p*-hydroxy phenylacetic acid, *γ*-hydroxybutyric acid, hydroxy-L-proline, L-leucine, L-alanyl-glycine, L-ornithine, L-phenylalanine, D-serine, D-galactonic acid lactone, D-alanine, L-threonine, D,L-carnitine, urocanic acid, *γ*-amino butyric acid, putrescine, uridine, phenyethylamine, 2-aminoethanol and 2,3-butanediol. The *mxaF* and *nifH* genes for, respectively, methanol dehydrogenase and nitrogenase activities are present in the genomic DNA of the strains REICA_082^T, REICA_032 and REICA_211. The DNA G+C contents of strains REICA_082^T and REICA_032 are 52.9 and 52.7 mol%, respectively. The 16S rRNA and *rpoB* gene sequences are deposited at the GenBank data bank under the accession numbers JF795011 and JF795017 for REICA_082^T, respectively.

The type strain, REICA_082^T (=NCCB 100390^T), was isolated from internal root tissues of rice (*Oryza sativa* L.) cultivar APO. The roots were sampled at flowering stage from an experimental paddy field at the IRRI, Philippines.

Table 5 Key reactions for biochemical differentiation of selected *Enterobacter* species

Taxa: 1, *Enterobacter oryziphilus* sp. nov. (n=3); 2, *Enterobacter oryzendophyticus* sp. nov. (n=3); 3, *E. radicincitans* (Kämpfer *et al*., 2005); 4, *E. turicensis* (Stephan *et al*., 2007); 5, *E. helveticus* (Stephan *et al*., 2007); 6, *E. pulveris* (Stephan *et al*., 2008); 7, *E. Arachidis* (Madhaiyan *et al*., 2010); 8, *E. cowanii* (Inoue *et al*., 2000); 9, *E. cancerogenus* (Dickey *et al*., 1988); 10, *E. oryzae* (Peng *et al*., 2009); 11, *E. cloacae* subsp. *cloacae* (Hormaeche & Edwards, 1958; Hoffmann *et al*., 2005); 12, *E. cloacae* subsp. *dissolvens* (Brenner *et al*., 1986; Wang *et al*., 2010); 13, *E. nimipressuralis* (Brenner *et al*., 1986); 14, *E. amnigenus* biovar 1 (Izard *et al*., 1981); 15, *E. gergoviae* (Brenner *et al*., 1980); 16, *E. pyrinus* (Chung *et al*., 1993; Stephan *et al*., 2008). The percentage of strains giving a positive result is scored as: -, 0–20%; v, 20–80%; +, 80–100%; ND, no data available; cell morphology: R, rods; CR, coccoid rods; SR, straight rods.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Voges-Proskauer (37°C)	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Methyl red test	+	+	-	+	+	+	-	-	-	+	-	-	+	-	-	-
Cell	SR	SR	R	CR	CR	CR	SR	R	SR	SR	R	R	R	SR	R	SR
Motility	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
B-Methyl-D-Glucoside	+	+	-	+	+	+	+	-	-	+	+	+	+	+	-	-
Ornithine decarboxylase	-	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+
Malonate decarboxylase	+	+	+	+	+	-	+	-	+	ND	-	V	-	+	+	+
Arginine dihydrolase	+	+	+	-	-	-	+	-	+	+	+	+	+	+	-	-
Aesculin hydrolysis	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+
Carbon source utilization																
Sucrose	+	+	+	-	-	+	+	+	-	+	+	+	-	+	+	+
D-Melibiose	-	V	-	+	+	+	-	+	-	+	+	+	+	+	+	+
Adonitol	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-
D-Sorbitol	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-
L-Fucose	+	V	ND	-	-	-	+	-	ND	+	-	-	-	-*	V*	-
L-Aspartic acid	+	-	+	+	+	+	+	ND	+	ND	ND	ND	ND	-	ND	ND
m-Inositol	+	+	-	-	-	-	+	-	-	ND	+	+	+	-	-	+
D-Arabitol	+	+	-	-	-	+	+	-	-	-	-	-	-	-	+	-
L-Rhamnose	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
D-Raffinose	-	+	-	-	-	+	+	+	-	ND	+	+	-	+	+	-
i-Erythritol	-	-	-	-	-	-	+	-	-	ND	-	-	-	-	-	ND

*data retrieved from Madhaiyan *et al*., (2010).

Chapter 6

Dynamics of rice endophytes – rise and fall of empires

Pablo R. Hardoim, Cristiane C.P. Hardoim, Leo S. van Overbeek, Jan D. van Elsas

Abstract

Bacterial endophytes are ubiquitous to virtually all terrestrial plants. With the increasing appreciation of studies that unravel the mutualistic interactions between plant and microbes, we increasingly value the beneficial functions of endophytes that improve plant growth and development. However, still little is known on the source of established endophytes as well as on how plants select specific microbial communities to establish associations. Here, we assessed the endophytic community of surface-sterilized rice seeds, encompassing two consecutive generations. We isolated members of nine bacterial genera. In particular, organisms affiliated with *Stenotrophomonas maltophilia*, *Mycobacterium abscessus* and *Ochrobactrum* spp. were isolated from both seed generations. PCR-DGGE profiles based on seed-extracted DNA revealed that approximately 45% of the bacterial community from the first seed generation was found in the second generation as well. We also recorded the dynamics of the rice endophytic communities from the seed up to tiller stage, using plants cultivated in sterile soils. In addition, we set up a greenhouse experiment with two soil types (low and neutral pH), two water regimes (flooded and unflooded) and three densities of a soil-derived community (low-, high- and un-inoculated). PCR-DGGE profiles performed with DNA extracted from different plant parts showed that the endophytic community structure was highly influenced by soil type, followed by water regime. Rice plants cultivated in neutral pH soil favoured the growth of *Pseudomonas oryzinhabitans* and *Rhizobium radiobacter*, whereas *Enterobacter oryzendophyticus* and *Dyella ginsengisoli* were dominant in plants cultivated in low pH soil. *Stenotrophomonas maltophilia* was the only conspicuous endophyte found in plants cultivated in both soils. Several members of the endophytic community originating from seeds were observed in the rhizosphere and surrounding soils. Their impact on the soil community is further discussed.

Introduction

Endophytes can be defined as microbial communities (bacteria and fungi) that are found inside plant tissue without causing any apparent harm to the host. Microbial endophytes have been reported in virtually all tissues of the host plant, including aseptically regenerated meristematic tissues of micropropagated plants (Dias et al., 2009; Lucero et al., 2008). The concept that seeds may serve as the sources of endophytes or pathogens was first launched by Baker & colleagues (1966). The presence of bacterial endophytes in, and dissemination from, seeds may be considered to represent an atypical event, which is certainly very difficult to demonstrate. However, the presence of bacteria has been documented in ovule tissues (several plants; Mundt & Hinkle, 1976), throughout seed maturing stages of rice (Mano et al., 2006) and in the endosphere of mature rice seeds (Kaga et al., 2009). Still, the concept of seeds as important sources of bacterial endophytes has been called controversial until recently (Mano & Morisaki 2008). A recent study revealed that a diverse array of endophytes could be obtained from plant tissue that once was considered germ-free, i.e. the callus tissue of micropropagated plants. The highly complex microbiome consisted of 11 bacterial and 17 fungal (ascomycete) taxa (Lucero et al., 2011). Furthermore, seed-borne endophytes improving seedling development were recently demonstrated in a study in which seed-borne *Pseudomonas* sp. SENDO 2, *Acinetobacter* sp. SENDO 1, and *Bacillus* sp. SENDO 6 improved cardon cactus growth by solubilising rock minerals (Puente et al., 2009a).

The bacterial community inside a plant is obviously prone to influences caused by changing plant physiology (Hallmann & Berg, 2006). Therefore, many factors that modify plant physiology, e.g. growth stage, soil type, agricultural management regime and even bacterial density, are thought to also promote significant shifts in the endophytic community structure. On the other hand, so-called competent endophytes might thrive even under adverse conditions (**Chapter 2**; Hardoim et al., 2008; Reiter et al., 2002). For the great majority of bacterial endophytes, their function or ecology inside the host plant is unknown. However, particular bacterial endophytes might actively influence the host physiology as a result of the production of phytohormones and/or the modulation of host ET levels. Many other PGP functions, such as fixation of N_2, solubilisation of inorganic P and provision of micronutrients, promotion of photosynthetic activity, induction of the plant defence system, production of antibiotics, biotransformation of heavy metals and biodegradation of organic pollutants, might also enhance host fitness (Compant et al.,

2010). These beneficial functions might be drastically improved when plant endophytes establish synergistic interactions (Glick et al., 2007; Taghavi et al., 2010).

In this study we present a comprehensive analysis of the bacterial endophytes of rice seeds by assessing the culture-dependent and -independent fractions of the bacterial community in two consecutive seed generations. Furthermore, we assessed the development of bacterial endophytes from second-generation seeds grown to plants in gamma-irradiated soils, at three and five weeks after seed germination. To gain insight into how environmental factors affect the bacterial endophytic community, we included different abiotic conditions, i.e. we used two soil types (neutral and low pH) and two water regimes (flooded and unflooded). We also assessed different biotic parameters, i.e. we introduced selected endophytes in two concentrations (low- and high-BID). We then assessed the bacterial communities that emerged in the bulk and rhizosphere soil, the root and shoot. We found that the seed-borne bacterial endophytes were highly diverse. As the plant developed, few of these became dominant while others were suppressed. The endophytic community in plant tissue was largely influenced by soil type, followed by water regime. These results suggest that, under our conditions of reduced soil microbial complexity, rice seeds are important sources of bacterial endophytes. Furthermore, plant physiology was found to play a major role in shaping the endophytic bacterial communities.

Material and Methods

Assessment of endophytic communities from seed endosphere

Rice (*Oryza sativa* L.) seed and seedlings from two consecutive generations were analyzed. Rice seeds from cultivar APO were obtained from IRRI (Los Baños, Philippines) and used for seed multiplication in greenhouse conditions at the University of Groningen, Netherlands. From hereon, seeds collected from IRRI and Groningen are referred to as first and second generations, respectively. Bacterial communities of the rice seed endosphere from both generations were assessed by culture-dependent and -independent approaches. Under aseptic conditions, the hulls were removed from the rice seeds (1 g) with sterilized forceps and immediately subjected to surface-sterilization with a solution (50 ml) containing 0.12% sodium hypochlorite (NaClO), salts (0.1 and 3% sodium carbonate and sodium chloride, respectively) and 0.15% sodium hydroxide (Hurek et al., 1994) at 30ºC for 25 min in orbital shaking (200 rpm). The sterilization procedure was followed by a

washing step to remove surface-adhered NaClO in 50 ml 2% sodium thiosulfate (Miche et al., 2001). This procedure was repeated twice at 30ºC for 10 min under orbital shaking (200 rpm) before the seeds were subjected to rehydration for 1h at room temperature in 100 ml autoclaved demineralised (demi-)water. In addition, to assess the endophytic communities from early seedling development, 15 surface-sterilized rice seeds from both generations were incubated on R2A medium (DB - Difco) for five days at 28ºC and then used to extract DNA from shoot, root and the remainder of the seed tissues. The endophytic communities from these samples were later compared to the seed communities.

Endophytic bacterial cells from surface-sterilized seeds and seedlings were released by disrupting the plant tissues with a soft-headed hammer as described (**Chapter 3**; Hardoim et al., 2011). The homogenates (100 µl) were used for serial tenfold dilutions, which were plated onto R2A, after which plates were incubated for one week at 28ºC. In addition, homogenates (1 ml) were used for DNA extraction following the protocol described by Hurek et al. (1994). For each 100 mg of plant material, 1.2 ml cell lysis solution was used, while phenol:chloroform (1:1 v/v) was used for deproteinization. The concentration and quality of the extracted DNA was assessed by electrophoresis in 1% agarose gels, followed by staining with ethidium bromide and visualization under UV light.

Dynamics of rice endophytes

Surface-sterilized rice seeds from the second generation were used to set up the experiment that assessed the endophytic bacterial community from root and shoot endospheres at three and five weeks after seed germination. The plants originating from the germinated seeds were cultivated in two soil types, i.e. Kollumerwaard – K, a clay loam soil with neutral pH (Groningen, The Netherlands) and Valthermond – V, a loamy sand soil with low pH (Drenthe, The Netherlands). Both soils were sterilized by applying gamma radiation (minimum 25 kGy, Isotron, Netherlands) and 500 g was aseptically transferred to polyester pots. Sterility of the soil was confirmed by plating, as soil suspensions prepared did not show any colony growth up to 15 days after being plated on R2A medium. Moreover, very faint (residual) bands were observed in PCR-DGGE profiles prepared with soil-extracted DNA.

For the experiment, both soils were watered to a final volume of 70% WHC with filter-sterilized (0.2 µm) 25%-strength Hoagland's nutrient solution. Five-day old rice seedlings that did no show any visible microbial outgrowth following (5-day) incubation on R2A medium at 28ºC, were individually transferred to sterile soils. Six replicates for each

treatment were used. Rice plants were cultivated in the greenhouse using a day/night cycle of 16/8h and 25/18ºC for light and temperature, respectively. Soil water was replenished daily to holding capacity with freshly prepared filter-sterilized 25%-strength Hoagland's nutrient solution.

Invasion assay

The invasion assay consisted of rice plants cultivated in the greenhouse and subjected to different abiotic and biotic treatments. Surface-sterilized rice seeds from second generation were cultivated in two soil types, i.e. K and V, subjected to two water regimes, i.e. watering and flooded, and exposed to three BID, i.e. uninoculated, low and high (10^4 and 10^7 bacterial cells g^{-1} soil, respectively).

The bacterial inoculum used consisted of a selected mixture of 15 previously-isolated rice root endophytes, i.e. *Enterobacter* sp. REICA_112, *Enterobacter oryziphilus* REICA_142^T, *Pseudomonas* sp. REICA_175, *Klebsiella* sp. REICA_034, *Aeromonas* sp. REICA_106 and REICA_164, *Herbaspirillum* sp. REICA_064, *Shewanella* sp. REICA_181, *Enterobacter oryzendophyticus* REICA_082^T, *Exiguobacterium* sp. REICA_016, *Micrococcus* sp. REICA_095, Alphaproteobacterium sp. REICA_149 and *Mycobacterium* sp. REICA_128, and three presumably competent endophytes used as controls, i.e. *Pseudomonas protegens* CHA0^T (Ramette et al., 2011), *Pseudomonas putida* P9 (Andreote et al., 2009) and *Burkholderia phytofirmans* RG44-4 (Sessitsch et al., 2005). Each strain was grown separately in R2A broth aerobically at 28°C with shaking (200 rpm). Bacterial cells were harvested in the exponential growth phase by centrifugation and washed twice with sterile PBS. Bacterial cells of each inoculum were combined with their respective amount of cells needed to achieve the final BID. The BID of each treatment was further confirmed using dilution plating on R2A medium. The mixed bacterial cells were diluted in filter-sterilized (0.2 μm) 25% Hoagland's nutrient solution, and added to the soil, establishing 70% of WHC of each soil. Filter-sterilized 25% Hoagland's nutrient solution was used in control treatment (uninocualted). Inoculated soils (500 g pot^{-1}) were covered with aluminum foil and incubated in the greenhouse for one week, for establishment of the bacterial communities, prior to the placement of five-day old rice seedlings in each. One seedling per pot and six replicates per treatment were used. Rice plants were then further cultivated in the greenhouse under the aforementioned conditions. At week three, after tiller formation, the plants exposed to low and high BID were subjected to flooding. At week five, the plants were harvested and the bacterial communities in soil free of roots

(denoted bulk soil), rhizosphere soil, the root and shoot tissues were assessed by a cultivation-independent approach. Individual rice plants were harvested and root-adhering soil particles were removed with a forceps and stored. Root and shoot tissues were segmented with a sterile scalpel and treated as individual sources of endophytes. Rice tissue surface sterilization was performed in 20-ml tubes filled with 10 ml sterilization solution as described above. Plant tissues were exposed (2 min) to NaClO solution and manually vortexed at room temperature. DNA from bulk and rhizosphere soils and plant tissues was extracted as described for seed samples. DNA from soil and rhizosphere was purified twice using the Wizard DNA clean-up system (Promega).

PCR-DGGE and ordination analyses

For PCR-DGGE analysis, the Chelius/Triplett nested PCR system (799F-1492R followed by 968F-1401R) was the most efficient approach to detect rice endophytic bacteria (Chelius & Triplett, 2001). DNA amplification conditions and PCR-DGGE analyses were performed as described previously (**Chapter 3**; Hardoim et al., 2011). The denaturing gradient gel was casted with a gradient of 40-55% denaturant (100% denaturant contained 7 M urea and 40% formamide) in a PhorU-2 apparatus, (Ingeny, Goes, Netherlands). The amplicons (150 ng) from each treatment with six replicates were loaded side-by-side in the same gradient gel and were cross-compared. Reference markers containing selected strains previously isolated from rice root endosphere (**Chapter 6**) were loaded at both edges and among treatments for normalization purposes. After the run, gels were stained with SYBR gold (Molecular Probes, Leiden, Netherlands) and the DGGE patterns were made visible by illumination with UV. The profiles were digitized using a digital camera and stored as TIFF files.

All PCR-DGGE profiles were analyzed using GelCompar II v 4.06 (Applied Maths, Sint-Martens-Latem, Belgium) as described previously (**Chapter 3**; Hardoim et al., 2011). Relative band intensity matrixes were exported and triplot ordination diagrams were generated by principal components analysis (PCA) of PCR-DGGE profiles using the package software CANOCO (Biometrics, PRI, Netherlands).

Isolates and PCR-DGGE band identifications

Rice seed endophytes were isolated using R2A at 28°C and replicated on the same medium to obtain pure cultures. Single colonies were used for identification by sequencing the partial 16S rRNA gene as described (Stevens & van Elsas, 2010). For this, the reverse primer 1401R was used in the sequencing reaction. Dominant bands from generated PCR-

DGGE profiles were selected for identification. Following excision, band DNA was extracted by incubating the polyacrylamide gel in 50 µl sterile TAE buffer solution for two days at 4ºC. From the homogenate, 2 µl was used as DNA template for PCR-DGGE re-amplification. PCR-DGGE bands with identical motility compared with the original PCR-DGGE pattern were subjected to identification by sequencing with reverse primer 1401R. In addition, 16S rRNA gene amplicons of rice seed endophyte strains were subjected to PCR-DGGE analysis and PCR-DGGE bands with identical denaturation motility were tentatively assigned to strains. The sequences obtained from the excised PCR-DGGE bands and the partial 16S rRNA gene from isolates were deposited in the GenBank under the accession numbers JN110430 to JN110462.

Results

Rice seed endophytic communities

The culturable endophytic community of rice seeds was assessed using the seeds from two consecutive generations. Seeds from first generation showed the highest population density, with 3.5 10^5 CFU g^{-1} FW, whereas the second generation revealed 4.5 10^3 CFU g^{-1} FW. The 16S rRNA gene identification of the seed-borne strains revealed that the endophytes encompassed members of nine genera within the classes *Alpha-* and *Gamma-proteobacteria, Flavobacteria, Bacilli* and *Actinobacteria* (Table 6). Strains closely related to *Stenotrophomonas maltophilia* (R2 and R8), *Mycobacterium abscessus* (R1 and R5) and *Ochrobactrum* spp (R3 – *O. tritici* and R12 – *O. grignonense*) were observed inside both seed generations. The seed endosphere strains R2, R6, R8, R9, R11, R12, R15 and R16 showed high 16S rRNA gene sequence similarity (> 99.0%) to bacteria isolated/sequenced from rice phytosphere, rhizosphere and paddy soil (Table 6).

PCR-DGGE analysis of the seed and rice tissue (5 days) endophytic communities revealed considerable complexity, with a total of 30 migration positions of the bands. The richness varied between 8 and 15 bands, which included six dominant bands that were erratically distributed in the midst of many faint ones (Fig. 17a). Seeds from the first and second generations revealed an almost equal richness, with, on average, respectively 9 and 8 PCR-DGGE bands. Five PCR-DGGE bands (Fig. 17a bands 9, 11, 12, R13 and one not identified) were shared in both generations. Shoots and roots of seedlings that originated from seeds of both generations showed slightly higher richness than that observed inside seeds, with respectively 13 and 11 PCR-DGGE bands on average. The endophytic

community shared in both generation of seedling shoot and root tissues encompassed, respectively, 29% (PCR-DGGE bands 9, 12, R13, R14 and one not identified) and 23% (bands 9, 11, 12, R13 and one not identified) of the total community. We tentatively identified 17 PCR-DGGE bands by sequencing (Table 7) and assigned three additional bands with identical motility behaviour to previously isolated seed endophytes (band identity is preceded by letter R, Fig. 17a). In the PCR-DGGE profile of seed and seedling endophytes, a total of 16 PCR-DGGE bands were identified, of which ten showed high 16S rRNA gene sequence similarity (> 99.0%) to bacteria isolated/sequenced from the root endosphere of mature rice plants growing in Philippines (Fig. 17a, PCR-DGGE bands 1, 2, 3, 4, 5, 7, 9, 10 and 14) and the rhizosphere of rice (Fig. 17a, band 12). PCR-DGGE bands 9, 12 and R13 were the most frequently found bands inside seeds and seedlings of both generations; they were closely related to *Stenotrophomonas maltophilia* (99.7% sequence similarity), *Pseudomonas protegens* CHA0^T (100%) and *Plantibacter flavus* DSM 14012^T (99.8%), respectively. The seed endophyte strains R8 and R6 showed the same motility behaviour of that found for PCR-DGGE bands 9 and 12, respectively. Two PCR-DGGE bands with identical motility (3 and 4, and 7 and 8) were identified as different species and further analyzed as pairs.

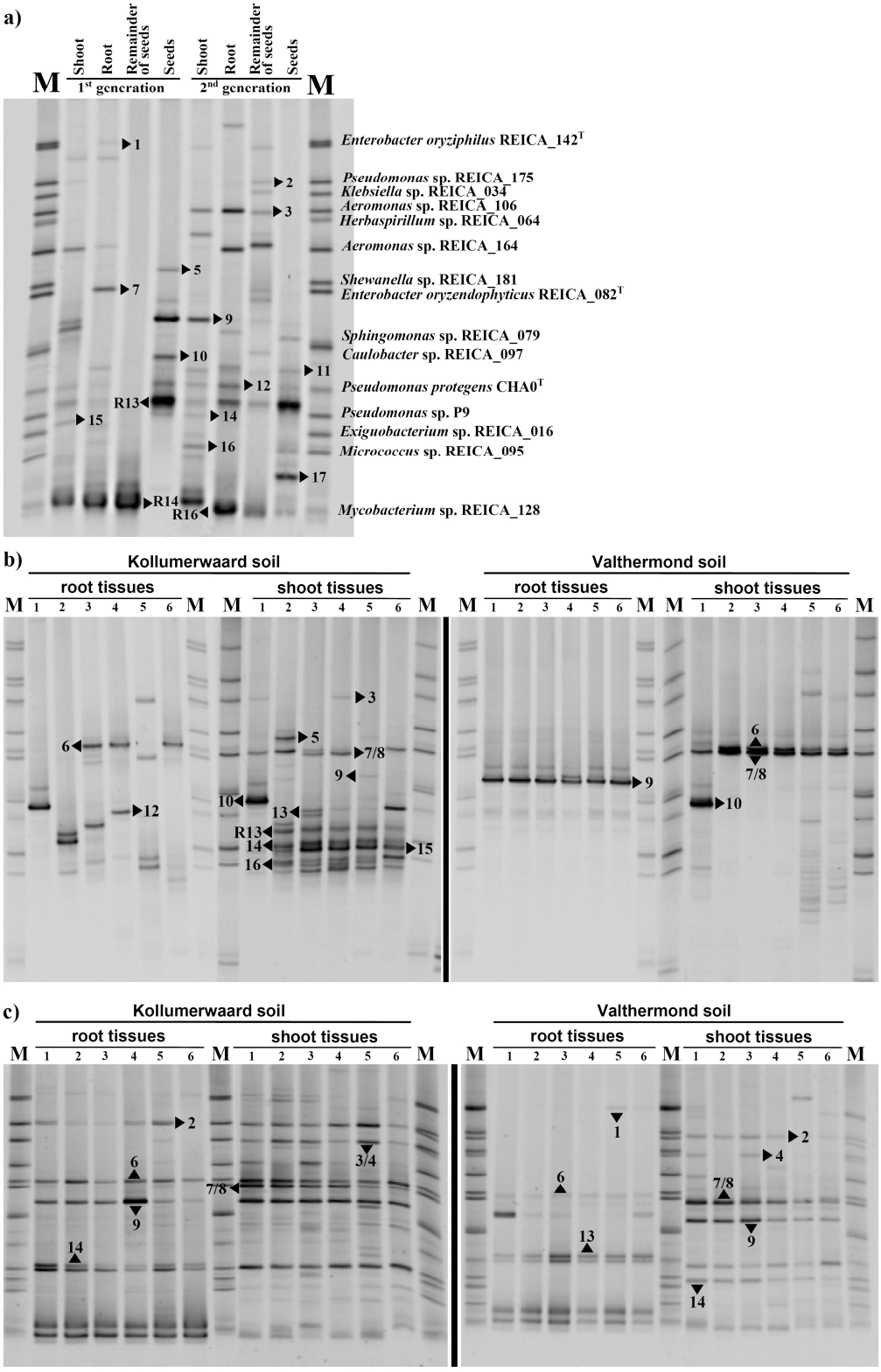

Fig. 17 Dynamics of rice endophytes

as revealed by PCR-DGGE profiles of seed tissues, three- and five-week-old rice plants. a) Rice endophyte PCR-DGGE patterns of surface-sterilized dehulled seeds and 5-day-old shoot, root and remainder of the seed tissues from two consecutive generations are shown. PCR-DGGE patterns of root and shoot endosphere community of three- b) and five- c) week-old rice plants cultivated in two soil types. Six replicates per treatments are shown. Arrow heads indicate identified communities (see Table 6 and 7), M – marker with a selection of 15 endophyte ribotypes.

Dynamics of rice endophytic community as revealed by plant development

As evidenced by PCR-DGGE, the endophytic bacterial community inside root and shoot tissues of three- and five-week-old rice plants cultivated in gamma-irradiated soils K and V revealed major differences between the soils in which the rice plants were cultivated (Fig. 17b and c). The richness of endophytes from plants cultivated in the K soil was higher than that found in V soil plants, independent of the plant tissue or time of analysis. The profile of the endophytic community from three-week-old plants cultivated on K soil showed 2 to 8 bands for root and 8 to 13 bands for shoot tissues, whereas plants cultivated on V soil harboured between 2 and 4 and 3 and 13 bands, respectively. Plants cultivated in K soil showed dominance of 5 bacterial communities (PCR-DGGE bands 7/8, R13, 14, 15, and 16) across shoot replicates, whereas the community structure from root tissues was erratically distributed across replicates (Fig. 17b). One PCR-DGGE band (9) was conspicuously present in all root samples of plants cultivated in V soil, whereas two bands (6 and 7/8) were dominant in the shoot tissues (Fig. 17b).

The PCR-DGGE profile of the endophytic community from five-week-old plants cultivated on K soil showed 4 - 7 bands in root tissues, of which four (bands 2, 6, 9 and 14) were conspicuous. In shoot tissues, 12 - 16 bands were found, of which 11 (PCR-DGGE bands 1, 2, 3, 6, 7, 9, 10, 14, 16 and two not identified) were conspicuous (Fig. 17c). The PCR-DGGE profile of plants cultivated in V soil showed 5 - 7 bands in the root tissues, of which two (bands 6 and 13) were conspicuous, and 6 - 11 were found in shoot tissues, from which four (bands 7/8, 9, 13, 14) were conspicuous.

The endophytic community of three- and five-week-old rice plants revealed high similarity with types found inside seeds and seedlings, with, respectively, 20 out of 24 and 19 out of 22 PCR-DGGE bands. Comparison of the endophytic communities during plant growth revealed diverse trends. For instance, in plants cultivated in K soil, the PCR-DGGE bands 2, 3/4, 9 and 14 were erratically found inside seedlings and three-week-old plant tissues but they became dominant in both tissues in the five-week assessment. Band 7/8 was exclusively dominant in shoot tissue. Band 6 was also dominant in the five-week samples, however it was never found inside seeds. Other PCR-DGGE bands (5, 10, 12, 13, R13, 15 and 16) found inside the seeds were erratically found in the three-week-old plants and not in the five-week samples. Others (11, 17, R14, R16) were only found in the seedlings. Plants cultivated in the V soil revealed different patterns, with PCR-DGGE bands 9 and 13 being conspicuously found across the replicates of three-week-old plants (only root tissues) and five-week-old plants (in both tissues), whereas band 1 (found in

seeds) was erratically found in five-week-old plants (in both tissues). PCR-DGGE bands 2, 3/4, 7/8, 10, 12, 14 and 16 were exclusively found in shoot tissues.

Factors affecting the endophytic community composition of rice

To obtain insight into how the endophytic community evolves in natural conditions, we designed an assay where we reduced the complexity of the system (i.e. rice growing in gamma-irradiated soil inoculated with 18 selected strains) and then assessed the bacterial community from four distinct habitats (i.e. bulk and rhizosphere soils, root and shoot endosphere tissues). As revealed by PCR-DGGE profiles, plants cultivated in K soil selected from members of seed-borne *Pseudomonas oryzihabitans* and inoculated *Aeromonas* sp. REICA_106 (only inoculated plants) for all habitats, whereas *Rhizobium radiobacter* was found in the rhizosphere soil, root and shoot tissues and inoculated *Pseudomonas putida* strain P9 was conspicuously found in the bulk and rhizosphere soils (Fig. 18). Plants from V soil selected for members of seed-borne *Enterobacter oryzendophyticus* and *Dyella ginsengisoli* for all habitats, whereas *Pseudomonas oryzihabitans* and *Pseudomonas putida* were restricted to shoot tissues, the inoculated *Enterobacter oryziphilus* REICA_142 to bulk soil and inoculated *Caulobacter* sp. REICA_097 to bulk and rhizosphere soils. The PCR-DGGE band 4 (*Herbaspirillum* sp. REICA_064) was found in the shoot tissues of inoculated treatments, independent of soil type (Fig 21 and 22), while the seed-borne band 9 (*S. maltophilia*) was found dominant in all assessed habitats for plants cultivated in K soil, whereas dominant in the shoot tissues and erratically distributed in the others habitats for plants cultivated in V soil (Fig. 18).

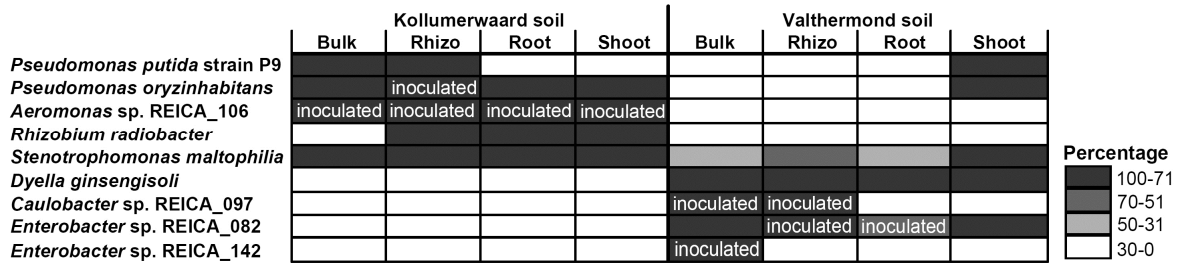

Fig. 18 Heat-map rice endophyte compostion in vary niches

Heat map composition of selected bacterial communities (rows) distributed in two soil types (K and V) and four different habitats (bulk and rhizosphere soil, root and shoot endosphere). Cell is shown in a spectrum of gray colour that correlates with percentage of observed species in a given habitat. Habitat in which the species was limited to inoculated treatments, the cell is shown with label "inoculated".

Using the collective data, we performed PCA for each habitat separately for each soil type (Fig. 19 and 20). Distribution of the rice shoot endosphere community in the PCA diagram was mainly influenced by water regime in both soil types, while an unclear division was observed for BID treatments in K and V soils, respectively (Fig. 19a and b). This suggested that both inoculation had exerted a minor effect on these bacterial communities. In the K soil, the endophytic bacterial communities from root tissues of plants cultivated in uninoculated soil was distributed along the second axis and differed from plants cultivated in low- and high-BID soil. In contrast, in the V soil the distribution of root endophytic communities from plants cultivated in uninoculated soil resembled those from inoculated plants (Fig. 19c and d).

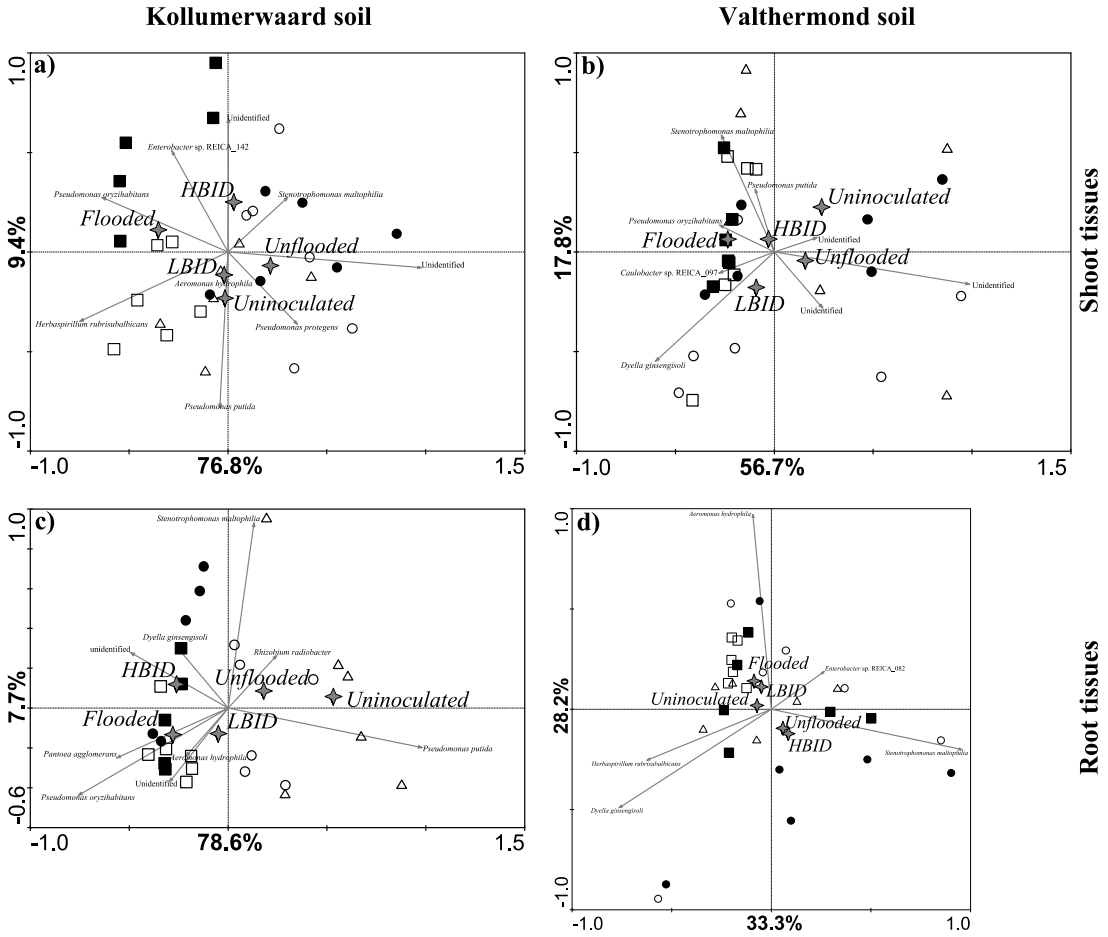

Fig. 19 Ordination diagrams of rice endophytic communities

Biplot ordination diagrams generated by PCA of PCR-DGGE profiles from endophytic community sampled from shoot (a and b) and root (c and d) tissues of plants cultivated on K (a and c) and V (b and d) soils. Squares and circle represent PCR-DGGE patterns of bacterial communities from plants submitted to, respectively, flooded and unflooded regimes and exposed to low- (empty square) and high- (full square) BID. Triangles represent PCR-DGGE patterns of bacterial communities from plants submitted to unflooded regime and cultivated on uninoculated soils. Six replicates of each treatment are shown. Stars represent nominal environmental variables. Arrows represent identified PCR-DGGE bands in which only the most descriptive communities are shown.

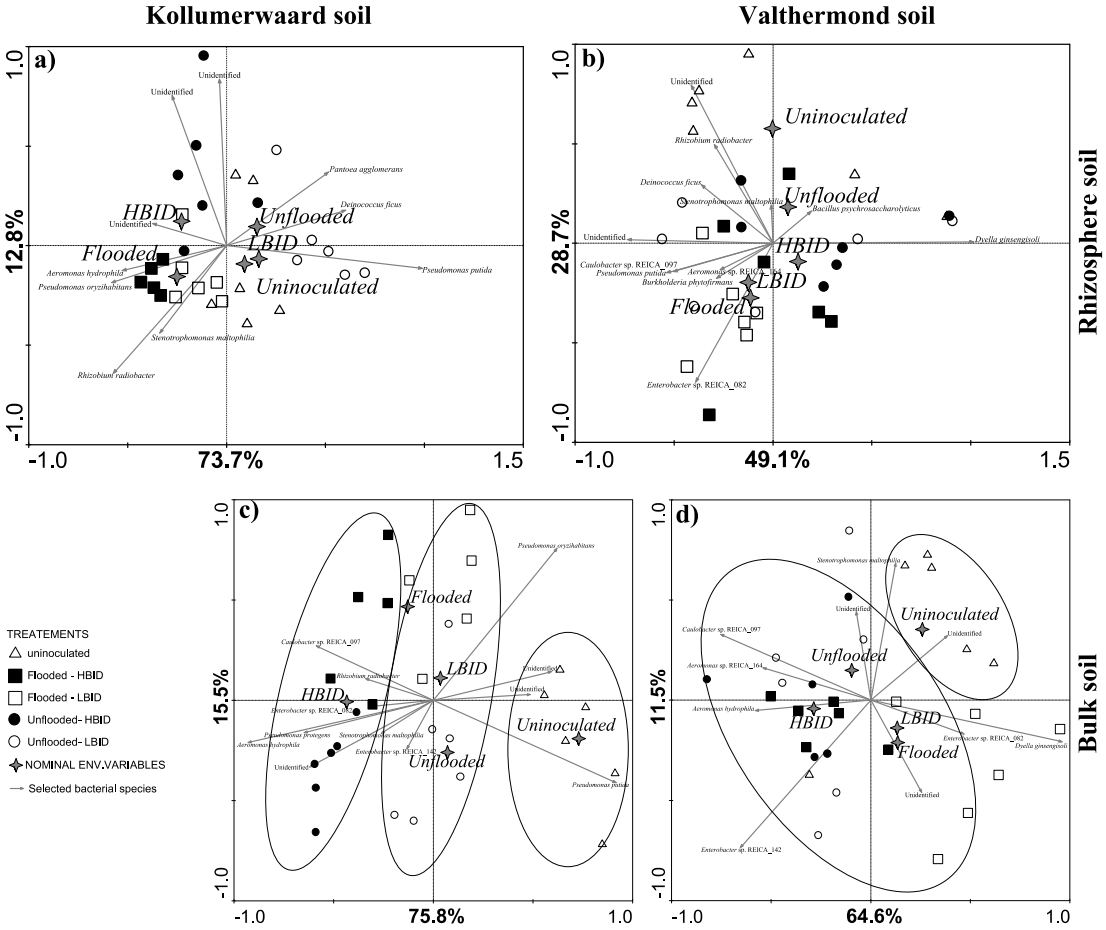

Fig. 20 Ordination diagrams of soil bacterial communities

Biplot ordination diagrams generated by PCA of PCR-DGGE profiles from bacterial community sampled from rhizosphere (a and b) and bulk (c and d) soils of plants cultivated on K (a and c) and V (b and d) soils. See Fig. 3 for symbol description.

In the rhizosphere, plants from inoculated V soil enhanced the community of the introduced bacteria *Enterobacter oryzendophyticus* strain REICA_082^T, *Burkholderia phytofirmans* strain RG44-4, *Aeromonas* sp. REICA_164, *Caulobacter* sp. REICA_097 and *Pseudomonas putida* strain P9, while plants from uninoculated soils selected for *Rhizobium radiobacter*, *Bacillus psychrosaccharolyticus*, *Stenotrophomonas maltophilia, Deinococcus ficus* and another as-yet-unidentified bacterium (Fig. 20b). In the K soil, the rhizosphere bacterial communities from plants cultivated in uninoculated soil was similar to plants cultivated in low-BID and both differs from those of high-BID. (Fig. 20a). Distribution of the soil community in the PCA diagram was mainly influenced by the BID factor in both soil types: in K soil, the bacterial communities from high-, low- and un-inoculated soil was distributed along the second axis and differed from each other in three main clusters (Fig. 20c). The soil communities from the uninoculated treatment showed growth of *P. oryzihabitans*, *P. putida* and two as-yet-unidentified bands, while the

introduced bacteria *Caulobacter* sp. REICA_097, *E. oryzendophyticus* strain REICA_082^T, *E. oryziphilus* strain REICA_142^T, *Aeromonas* sp. REICA_106 and *P. protegens* strain CHA0^T were enhanced after inoculation. In the V soil, the PCA diagram revealed two main clusters (Fig. 20d), which were separated along the second axis. The bacterial communities from the high-BID soil were distributed amongst the low-BID ones. Both revealed enhanced growth of *Caulobacter* sp. REICA_097, *E. oryzendophyticus* strain REICA_082^T, *E. oryziphilus* strain REICA_142^T, *Aeromonas* sp. REICA_106, *Aeromonas* sp. REICA_164, *Dyella ginsengisoli* and one unidentified bacterium, whereas the community from uninoculated soil differed from the previous and enhanced the growth of *S. maltophilia* and two unidentified bacteria.

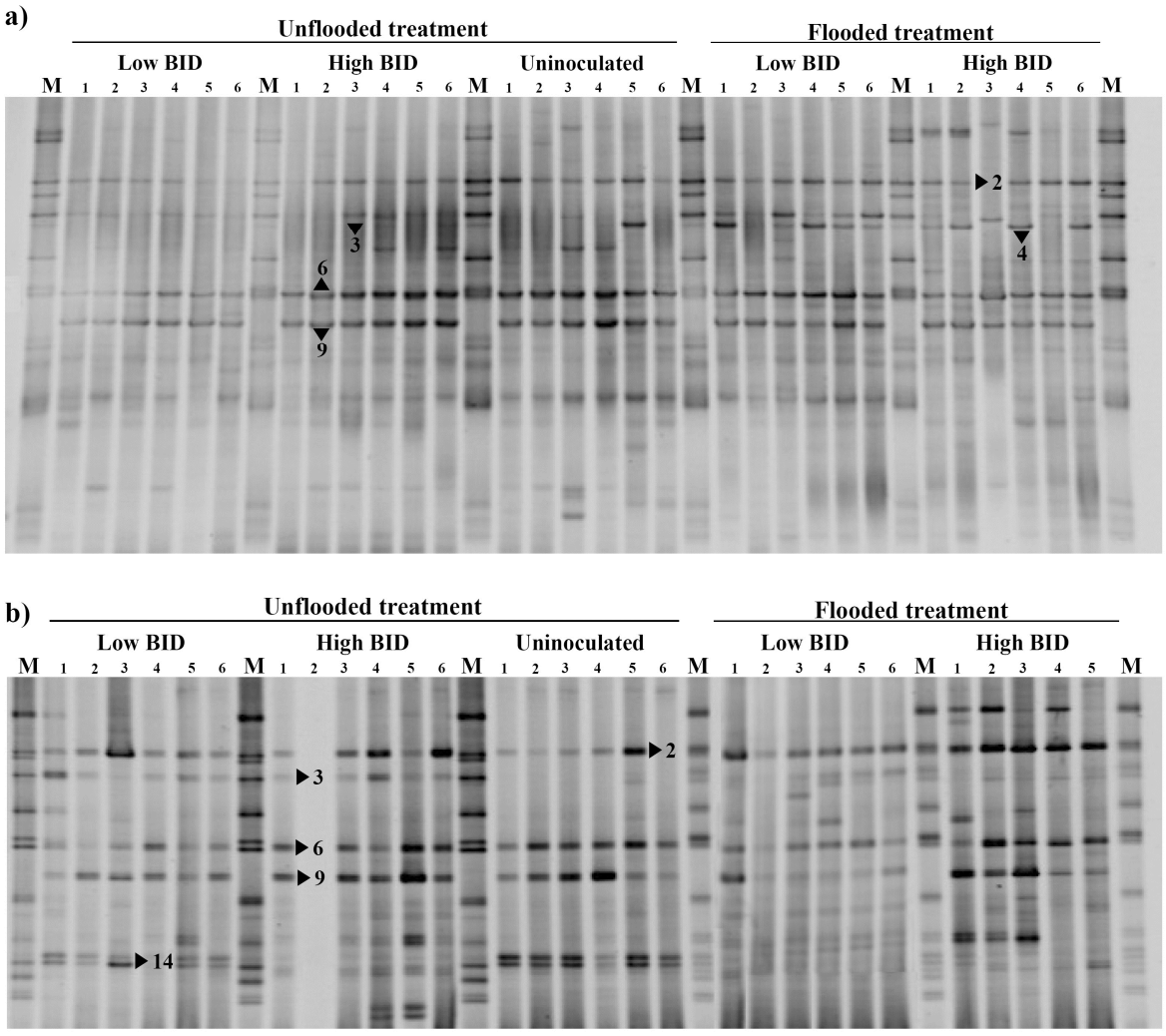

Fig. 21 PCR-DGGE profiles of rice endophytes in K soil

PCR-DGGE profiles of shoot a) and root b) endosphere community of rice plants cultivated in K soil. Rice plants were subjected to unflooded and flooded regimes and exposed to low-, high- and un-inoculated treatments. Six replicates per treatments are shown. Arrow heads indicate identified communities (see Table 6 and 7).

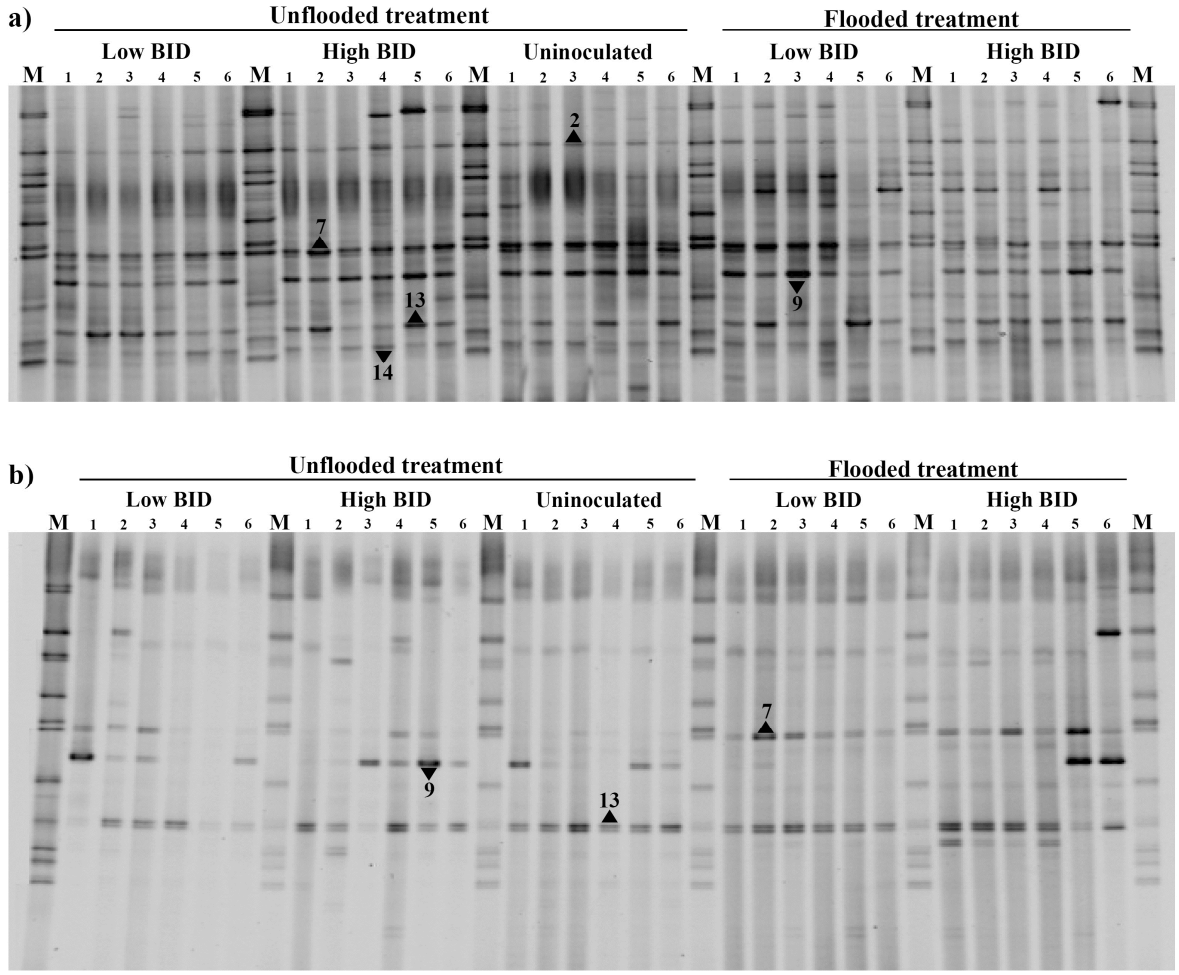

Fig. 22 PCR-DGGE profiles of rice endophytes in V soil

PCR-DGGE profiles of shoot a) and root b) endosphere community of rice plants cultivated in V soil. Rice plants were subjected to unflooded and flooded regimes and exposed to low-, high- and un-inoculated treatments. Six replicates per treatments are shown. Arrow heads indicate identified communities (see Table 6 and 7).

Discussion

This paper clearly showed that seeds are undoubted a source of endophytic bacteria that come up in the early rice growth stages when plants are cultivated in soil deprived of a bacterial community. There are at least three major pieces of evidence to support this contention, as listed below.

I) Many of the rice seed-borne endophytes that were found were closely related to bacteria that have previously been isolated from inside maturing and mature rice seed tissues (Mano et al., 2006 and Cottyn et al., 2009), the endosphere of rice root (**Chapter 4**) and shoot tissues (Mano et al., 2007), the rhizosphere of rice (Steindler et al., 2008) and wheat plants (Lebuhn et al., 2000), the phyllosphere of

grasses (Behrendt et al., 2002), hay dust (Kämpfer et al., 2000) and soil in which rice had been cultivated (Shrestha et al., 2007).

II) Throughout plant development, shoot tissues showed higher richness than root tissues. Plants cultivated in open fields often reveal the opposite observation, with higher bacterial richness in the root tissues (Hallmann et al., 1997). Mano & colleagues (2007) observed that the endophytic community in leaves of rice plants cultivated in the open field was similar to that found from seed tissues and differed drastically from that inside root tissues. The results suggested that rice seed endophytes are adapted to plant tissue and rapidly colonize shoots, in which there is less competition than in the root, which is surrounding by soil bacterial communities.

III) The assessment of the bacterial community from internal plant tissues to surrounding soils at plants cultivated in uninoculated and inoculated soils showed similar endophytic communities, which differed in the rhizosphere and were unrelated to those in the soil.

Given the potential of endophytes to spread inside the host, it is not surprising that plant seeds serve as a vector for dissemination of bacteria. In fact, a large diversity (284 genomic FPTs determined by BOX-PCR) was observed in a study on the bacterial communities of rice seeds (Cottyn et al., 2009). The authors showed that a great majority of the isolates was correlated to the sampling site where the seeds derived from. Only a few, such as *Enterobacter cloacae*, *Pseudomonas oryzihabitans* and *Curtobacterium* spp., were detected on the seeds obtained from all sampling sites (i.e. 12 sites), whereas others, including *Rhizobium radiobacter*, *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Herbaspirillum rubrisubalbicans* and *Microbacterium* spp., were isolated from seeds collected in more than one (but not all) sampling site. In addition, strains closely related to *Rhizobium radiobacter*, *Pseudomonas oryzihabitans*, *Aeromonas* sp., *Bacillus* spp., *Sphingomonas* sp., *Acinetobacter* sp. *Curtobacterium* sp., *Enterobacter* sp., *Microbacterium* sp., *Pantoea* sp., *Paenibacillus* sp. and *Ochrobactrum* sp. have also been isolated from the internal tissues of soybean seeds (Oehrle et al., 2000; Assumpção et al., 2009). These results suggest that a vast number of bacteria are specialized to seed environments and can be found even in different host species. It has been demonstrated that vertical transmission of beneficial bacterial endophytes might increase host fitness upon adverse environmental conditions (Puente et al. 2009) or under conditions without induced stress (Mastretta et al., 2009). Therefore, it is tempting to assume that seeds

harbour diverse numbers of endophytes that might become important, differentially in accordance with the local conditions, for the development of the new host.

In our study, we have isolated *S. maltophilia* from the rice seeds in two consecutive generations and have also shown that this bacterium competitively colonizes the tissues of the next generation plants, spreading into the rhizosphere and even out of the rhizosphere into the surrounding soil. *S. maltophilia* is ubiquitous in the environment and is commonly isolated worldwide from clinical specimens and environmental sources (i.e. mainly water, soil and plants). Genotypic and phenotypic analyses of 40 selected strains of *S. maltophilia* revealed high intraspecies diversity, which was not correlated to the sources of isolation (Berg et al., 1999). Various plant-beneficial properties, such as the production of the phytohormone IAA and siderophore, fixation of N_2, oxidation of elemental sulphur, production of antibiotic, VOCs with antifungal activity and hydrolytic enzymes, combined with great metabolic versatility, osmotic protection and heavy metal tolerance, places such strains of *S. maltophilia* as promising candidates for plant growth promotion. This, especially on marginal soils used for phytoremediation or soils supporting plant disease development (Ryan et al., 2009). Many strains of *S. maltophilia* have been isolated from rhizosphere and endosphere of various plants (Hayward et al., 2010); when inoculated, they were shown to enhance plant biomass production in corn (Mehnaz et al., 2010), sorghum (Idris et al., 2009), canola (de Freitas et al., 1997), potato (Sturz et al., 2001) and poplar trees (van der Lelie et al., 2009) cultivated under greenhouse conditions. Although the genome analysis of *S. maltophilia* R551-3 revealed many genes dedicated to motility, adaptation to and colonization of plant host tissues (Taghavi et al., 2009), our results showed that *S. maltophilia* is transmitted via seeds and can spread out of the host invading the rhizosphere and surrounding soils. The results suggest that *S. maltophilia* is highly adapted to the plant environment and that the strategy to interact with plants might be the outcome of both dissemination and colonization.

Here, the endophytic community of rice was mainly influenced by soil type, where rice plants cultivated in K soil, a neutral pH soil, showed higher richness and were extensively colonized by *P. oryzihabitans* and *R. radiobacter*, whereas plants cultivated in V soil, an acid soil, favoured the growth of *E. oryzendophyticus* and *D. ginsengisoli*. Members of these bacteria have been isolated from seeds and/or the phytosphere of various plants (Anandham et al., 2008; Cottyn et al., 2009; Hallmann and Berg, 2006; Oehrle et al., 2000), suggesting that they might have a long history of relationship with host plants. These possibly intimate interactions might be the result of beneficial mutualism, e.g.

strains of *P. oryzihabitans* containing ACC deaminase (strain Ep4; Belimov et al., 2001), or capable of solubilising inorganic phosphate (strain B4M-K; Collavino et al., 2010), production of IAA, siderophore and fixation of N_2 (G6; Loaces et al., 2011) have been reported to increase host biomass. Occasionally, commensalism might come into play, e.g. the plant-associated *R. radiobacter* (formerly *Agrobacterium tumefaciens*) is the causal agent of crown gall in dicotyledons, however it showed limit pathogenicity towards monocotyledons (de Cleene, 1985). In our previous study, the assessment of root endophytes from mature rice plants cultivated in field soil revealed that members of *Enterobacter* were the most abundant and the most genetically diverse (**Chapter 4**). Therefore, it is not surprising that members of *Enterobacter* might be transmitted via seeds. Although we have not isolated any *Enterobacter* strain in our study, we identified two PCR-DGGE bands from second seed generation profiles that were closely related to the previously found *Enterobacter* members. The recently-described *D. ginsengisoli* was initially isolated from a ginseng field in South Korea (Jung et al., 2009). *D. ginsengisoli* strain ATSB10, containing ACC deaminase and with ability to solubilise inorganic P and to produce β-1,3 glucanase, has been reported to increase by 145% the root length of canola seedlings (Anandham et al., 2008). The relationship of *D. ginsengisoli* with rice plants is unknown and this study is the first documentation that they may be associated.

In summary, seeds from rice plants harbour a great diversity of bacteria that, in response to plant physiological status, can become competent endophytes. Some organisms might even spread out into rhizosphere and surrounding soil, therefore directly interacting with soil microbial communities (Raaijmakers et al., 2009). Furthermore, due to their metabolic versatility, seed-borne bacterial endophytes might also increase the fitness of plants, giving the host a competitive advantage over other (indigenous) plant communities (Klironomos, 2002). This may affect whole-ecosystem functioning (Himler et al., 2011). Our data suggest that under reduced habitat complexity, this assumption may be met. It remains an open question whether seed-borne endophytes are selected by the host to increase the fitness of the next generations or bacterial endophytes use seeds as vector for dissemination and colonization of new environments.

Table 6 Identification of isolated strains from this study

Strains[a]	Accession number	Closest type strain (accession number)	Similarity (%)	Closest rice associated bacteria (accession number)	Similarity (%)	Sources[b]
R6	JN110435	*Pseudomonas saponiphila DSM 9751T (FM208264)*	720/723 (99.6)	*Pseudomonas* sp. MDR7 (AM911672)	723/723 (100)	R
R2	JN110431	*Stenotrophomonas maltophilia IAM 12423T (AB294553)*	789/792 (99.6)	Uncultured *Stenotrophomonas* clone SHCB1148	785/792 (99.1)	RE1
R8	JN110437	*Stenotrophomonas maltophilia IAM 12423T (AB294553)*	662/663 (99.8)	Uncultured *Stenotrophomonas* clone SHCB1148	661/663 (99.7)	RE1
R3	JN110432	*Ochrobactrum tritici SCII 24T (AM114402)*	741/741(100)	*Ochrobactrum* sp. RFNB9 (FJ266319)	727/741 (98.1)	PF
R12	JN110441	*Ochrobactrum grignonense OgA9aT (AJ242581)*	754/755 (99.9)	*Ochrobactrum* sp. RFNB9 (FJ266319)	749/755 (99.2)	PF
R7	JN110436	*Sphingomonas yanoikuyae IFO 15102T (D13728)*	717/721 (99.4)	Uncultured *Sphingomonas* clone SHCB0924	696/723 (96.3)	RE1
R11	JN110440	*Flavobacterium johnsoniae DSM 2064T (AM230489)*	608/619 (98.2)	*Flavobacterium* sp. P-135 (AM412169)	615/620 (99.2)	PS
R4	JN110433	*Paenibacillus humicus PC-147T (AM411528)*	547/590 (92.7)	*Paenibacillus* sp. RFNB4 (FJ266315)	542/588 (92.2)	PF
R10	JN110439	*Agromyces mediolanus DSM 20152T (X77449)*	674/674 (100)	*Curtobacterium* sp. Pd-E-(s)-l-D-6(4) (AB242985)	198/204 (97.1)	SE
R9	JN110438	*Curtobacterium citreum DSM 20528T (NR_026156)*	720/721 (99.8)	*Curtobacterium* sp. Pd-E-(l)-e-D-1(4) (AB291847)	203/203 (100)	LE
R16	JN110445	*Curtobacterium herbarum DSM 14013T (AM410692)*	798/800 (99.7)	*Curtobacterium* sp. Pd-S-(l)-l-D-3(6) (AB291903)	248/250 (99.2)	LS
R14	JN110443	*Frigoribacterium faeni DSM 10309T (AM410686)*	717/719 (99.7)	*Curtobacterium* sp. Pd-E-(l)-e-D-3(5) (AB291849)	194/199 (97.5)	LE
R15	JN110444	*Microbacterium oleivorans DSM 16091T (AJ698725)*	791/797 (99.2)	*Microbacterium* sp. Pd-S-(l)-l-D-6(16) (AB291906)	311/311 (100)	LS
R1	JN110430	*Mycobacterium abscessus CIP 104536T (AY457071)*	574/576 (99.6)	*Mycobacterium* sp. Pd-E-(r)-m-D-6(5) (AB291833)	329/343 (95.9)	RE2
R5	JN110434	*Mycobacterium abscessus CIP 104536T (AY457071)*	622/623 (99.8)	*Mycobacterium* sp. Pd-E-(r)-m-D-6(5) (AB291833)	308/322 (95.6)	RE2
R13	JN110442	*Plantibacter flavus DSM 14012T (AJ310417)*	629/630 (99.8)	*Microbacterium* sp. P-65 (AM411961)	615/631 (97.5)	PS

[a] Rice strains isolated from first (R1-R4) and second (R5-R15) generation seeds.

[b] Source of the closest rice associated bacteria, LE – Leaf Endophyte (Mano et al., 2007); LS – Leaf surface (Mano et al., 2007); PF – Paddy Field (Islam et al., unpublished); PS – Paddy Soil (Shrestha et al., 2007); R - Rhizosphere (Steindler et al., 2008); RE1 - Root Endosphere (**Chapter 4**); RE2 - Root Endosphere (Mano et al., 2007) and SE – Seed endophyte (Mano et al., 2006).

Table 7 Identification of excised PCR-DGGE bands

DGGE band ID	Accession number	Closest type strain or known strain (accession number)	Similarity (%)	Closest rice associated bacteria (accession number)	Similarity (%)	Sources[a]
1	JN110446	*Enterobacter oryziphilus* REICA_142^T (JF795013)	382/382 (100)	*Enterobacter oryziphilus* REICA_142^T	382/382 (100)	RE1
2	JN110447	*Pseudomonas oryzihabitans* IAM 1568^T (AM262973)	379/380 (99.7)	*Pseudomonas* sp. REICA_175	379/380 (99.7)	RE1
3	JN110448	*Aeromonas hydrophila* subsp. *dhakensis* LMG 19562^T (AJ508765)	371/373 (99.5)	*Aeromonas* sp. REICA_106	373/373 (100)	RE1
4	JN110449	*Herbaspirillum rubrisubalvicans* ICMP 5777^T (AF137508)	346/349 (99.1)	*Herbaspirillum* sp. REICA_064	346/349 (99.1)	RE1
5	JN110450	*Acinetobacter beijerinckii* LUH 4759^T (AJ626712)	382/382 (100)	Uncultured *Acinetobacter* clone SHCB0621	381/382 (99.7)	RE1
6	JN110451	*Rhizobium radiobacter* IAM 12048^T (AB247615)	378/383 (98.7)	Uncultured *Rhizobium* SHCB0425	369/386 (95.6)	RE1
7	JN110452	*Enterobacter oryzendophyticus* REICA_082^T (JF795011)	376/376 (100)	*Enterobacter oryzendophyticus* REICA_032^T	376/376 (100)	RE1
8	JN110453	*Escherichia coli* O111:H str. 11128 (AP010960)	382/382 (100)	*Enterobacter* sp. REICA_128	378/382 (98.9)	RE1
9	JN110454	*Stenotrophomonas maltophilia* IAM 12423^T (AB294553)	382/383 (99.7)	Uncultured *Stenotrophomonas* SHCB1148	382/383 (99.7)	RE1
10	JN110455	*Pantoea agglomerans* DSM3493^T (AJ233423)	380/380 (100)	Uncultured *Pantoea* SHCB0588	378/380 (99.5)	RE1
11	JN110456	*Neisseria meningitidis* M01-240149 (CP002421)	374/375 (99.7)	Uncultured bacterium clone J-3FECA52 (DQ340883)	291/308 (94.5)	RE2
12	JN110457	*Pseudomonas protegens* CHA0^T (AJ278812)	378/378 (100)	*Pseudomonas* sp. MDR7 (AM911672)	378/378 (100)	R
13	JN110458	*Dyella ginsengisoli* Gsoil 3046^T (AB245367)	373/373 (100)	*Dyella* sp. V-6.1 (JF429979)	367/373 (98.4)	PF
14	JN110459	*Pseudomonas putida* BIRD-1 (CP002290)	378/378 (100)	Uncultured *Pseudomonas* SHCB0777	378/378 (100)	RE1
15	JN110460	*Bacillus psychrosaccharolyticus* S156^T (AY509230)	373/379 (98.4)	*Bacillus* sp. P-150 (AM412171)	367/381 (96.3)	PS
16	JN110461	*Deinococcus ficus* CC-FR2-10^T (AY941086)	377/379 (99.5)	Uncultured bacterium clone J-3FECC29 (DQ340907)	266/293 (90.8)	RE2
17	JN110462	*Achromobacter spanius* LMG 5911^T (AY170848)	367/374 (98.1)	Uncultured bacterium clone J-3FECC48 (DQ340912)	365/374 (97.6)	RE2

[a] Source of the closest rice associated bacteria: PF – Paddy Field (Cuong et al., 2011); PS – Paddy Soil (Shrestha et al., 2007); R - Rhizosphere (Steindler et al., 2008); RE1 - Root Endosphere (**Chapter 4**) and RE2 - Root Endosphere (Sun et al., 2008).

Chapter 7

Metagenome analysis of endophytes in rice roots reveals a highly adapted community[1]

Angela Sessitsch, Pablo Hardoim, Jessica Döring, Alexandtra Weilharter, Andrea Krause, Tanja Woyke, Brigit Mitter, Lena Hauberg, Frauke Friedrich, Monali Rahalkar, Thomas Hurek, A. Sarkar, Levente Bodrossy, Leo van Overbeek, Darshan Brar, Jan D. van Elsas, and Barbara Reinhold-Hurek

Abstract

Roots are the primary site of interaction between plants and microorganisms. To meet food demands in changing climates, improved yields and stress resistance are increasingly important, stimulating efforts to identify factors that affect plant productivity. The role of bacterial endophytes that reside inside plants remained largely unexplored, as analysis of their specific functions is impeded by difficulties to cultivate most prokaryotes. Here we present the first metagenomic approach to analyze an endophytic bacterial community resident inside roots of rice, one of the most important staple foods. Metagenome sequences were obtained from endophyte cells extracted from roots of field-grown plants. Putative functions were deduced from protein domains or similarity analyses of protein-encoding gene fragments, and allowed insights in capacities of endophyte cells. For habitat-specific fingerprints, quantitative gene content analyses were compared between several different metagenomes based on the relative abundances of protein-coding genes. This allowed us to predict traits and metabolic processes important for the endophytic lifestyle, suggesting that the endorhizosphere is an exclusive microhabitat requiring numerous adaptations in comparison to the soil environment. Abundant features included flagella, plant-polymer-degrading enzymes, protein secretion systems, iron acquisition and storage, quorum sensing, and detoxification of ROS. Surprisingly, endophytes might be involved in the entire nitrogen cycle, as protein domains involved in N_2-fixation, denitrification and nitrification were detected and selected genes even expressed. Our data suggest a high potential of the endophyte community for plant-growth promotion, improvement of plant stress resistance, biocontrol against pathogens and bioremediation, irrespective of their culturability.

[1] This manuscript was done in collaboration with the co-authors and this version was submitted to the ISME journal.

Introduction

Rice is the staple food for the largest number of people on earth, and rice cultivation is resource-intensive. Irrigated rice production consumes annually about 10% of global fertilizer N production and approx. 40 % of developed water supplies in Asia, however also contributes to greenhouse gas emissions by microbial conversions. The competition for arable land and water and the rise in rice consumption demand increased yields and improved management practices (FAO, 2008). Particularly endophytes that reside inside tissues of healthy plants are likely to positively affect the host (Weyens et al., 2009b), however, the potential of applying endophytic bacteria is still underexplored (Mano & Morisaki, 2008). Rice roots harbor endophytes (Hurek et al., 1994), which can reach up to 108 cultivable N2-fixing bacteria per g of root dry weight (Barraquio et al., 1997) and even larger numbers of bacteria that have defied cultivation so far (Engelhard et al., 2000; Knauth et al., 2005; Mano & Morisaki, 2008). Thus endophytes are likely to considerably affect their host plant.

To understand and manipulate their contribution, it is important to decode metabolic processes, adaptations and beneficial characteristics. However, assessing microbial functions is impeded by difficulties to cultivate most prokaryotes, and endophytes inside host tissues are not easily amenable to biochemical or genetic analyses. Our knowledge on functions, niche adaptations, host-microbe interactions and putative beneficial traits so far depends on cultivated endophytes from rice (Krause et al., 2006; Yan et al., 2008) and other plants (Compant et al., 2010). As cellular capacities of uncultured microbial communities can be deciphered using metagenomic approaches (Dinsdale et al., 2008), we applied this strategy to investigate for the first time in a culture-independent manner key biological processes contributed by an endophytic bacterial community in roots of *Oryza sativa*. Analysis of protein-coding genes allowed identification of metabolic and interactive potential of the endophyte community. In addition, we performed a gene-centric comparative analysis (Tringe et al., 2005) of different metagenomes based on the relative abundances of protein-coding genes and mRNA expression analysis for selected bacterial genes.

Methods

Extraction of endophyte cells from rice roots

Rice variety *O. sativa* cv. APO (IR55423-01) was grown on three plots at IRRI, Los Baños, Philippines, with N-P-K fertilizer at 90 – 30 – 30 kg per ha. Root samples from flowering plants were taken within two weeks (September/October 2006), processed daily for endophyte extraction, and cells were pooled for metagenome analysis. Roots were thoroughly washed with tap water and small lateral roots were removed. The rhizoplane was chemically sterilized (approx. 5% sodium hypochlorite for 2 min), and rhizoplane bacteria were physically removed by established methods (Reinhold et al., 1986) using vigorous shaking with sterile glass beads in sterile water. Roots depleted of surface bacteria were then disrupted by scalpel in order to release the endophytes, and then shaken again with glass beads in sterile saline (0.9% NaCl) for 4 h at 30°C to detach microorganisms. Large plant and fungal cells and iron particles were removed from the supernatant by filtration (5 µm pore size), and residual cells pelleted at 4°C at 15.000 x g, and stored in liquid nitrogen.

Extraction and purification of genomic DNA

Endophyte cells were suspended in 200 mM Tris-HCl pH 8.5, 25 mM EDTA (ethylenediaminetetraacetic acid) and 1% SDS, and the lysis of the cells was performed by bead-beating (FastPrep instrument, BIO 101, La Jolla, CA). The suspension was incubated with Proteinase K for one h at 60°C, extracted with phenol/chloroform/isoamylalcohol, and nucleic acids were precipitated with ethanol and dissolved in TE (pH 8.0; Tris-EDTA). After purification by gel filtration and RNase One treatment for 2 h at 25°C, the DNA was again extracted with phenol/chloroform/isoamylalcohol, precipitated with ethanol and resuspended in TE buffer (pH 8.0).

Analysis of nifH pools and bacterial transcripts

For mRNA analysis and DNA-based clone library construction, roots were briefly washed and shock-frozen in liquid nitrogen. Root total DNA for profiling (Engelhard et al., 2000) or RNA and DNA for clone libraries (Hurek et al., 2002; Knauth et al., 2005) were essentially extracted as previously described after homogenization of roots in liquid nitrogen and cell disruption by bead beating. Amplification of *nifH* fragments (Hurek et al., 2002) was slightly modified by using cDNA from reverse transcription (1 µl of 50 µl) for amplification in a separate reaction (MolTaq, Molzyme, Bremen, Germany). Either T-RFLP (Terminal Restriction Fragment Length Polymorphism) analysis (Knauth et al.,

2005) was performed after electroelution of fragments from agarose gels, or clone libraries were constructed. *nifH* fragments were cloned into the pJET 1.2 vector using the CloneJetTM PCR cloning kit (Fermentas, Germany). Sequence analysis of 70 clones included rarefaction analysis (PAST program, http://folk.uio.no/ohammer/past/), construction of a phylogenetic tree based on *nifH* protein sequences from related cultured and uncultured diazotrophs, and in-silico restriction analysis of the clones.

The following primers were designed based on metagenomic sequences and used for reverse transcription (RT) or amplification by PCR. Underscored positions represent LNA (locked nucleic acid) modifications to improve the sensitivity of reverse transcription (Burbano et al., 2010). *nirK*, genes related to nitrite reductase genes of *Bradyrhizobia*, NirK_RT_Brady_96 TTCACCTGGGTCATCAGAT; NirK_f_Brady_96 GACGAGAAGGGCAATTTC; NirK_r_Brady_96 ACTTGCCTTCGACCTTGAA. *fliC*, gene for gammaproteobacterial flagellin, FliCmet_RT GGMASCTGGTTGGCCTG, FliCmet_f TGGGTGCMTCSCAGARCCG, FliCmet_r GCCGGCTATGCGMGCGG. *gst*, gene for glutathione S-transferase, GST_f CTGGAAGGCCAAGACCAAC; GST_r ACCAGATCTTGACCGAGG. *nirK*, gene for bradyrhizobial-type nitrite reductase, nirK-RTBrady-96 TTCACCTGGGTCATCAGAT; nirK-f-Brady-96 GACGAGAAGGGCAATTTC; nirK_r_Brady_96 ACTTGCCTTCGACCTTGAA. *amoA*, gene for gammaproteobacterial ammonium monooxygenase, AmoA_gamma_RT GCCGAMGCRGTCACCATCAA; AmoA_f_gamma GTTCCKGCKGCRCTGTTGC; AmoA_r_gamma CCAGGTKCCGGTCGTTCC. After reverse transcription for 30 min at 47°C, conditions for amplification were: for *fliC*, an initial denaturation at 94°C for 1 min, then 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, extension for 30 s at 72°C, with a final extension for 5 min at 72°C were used. For *gst*,, annealing was modified to 56°C. For *nirK*, initial denaturation was done at 95°C for 5 min, then 40 cycles of denaturation for 1 min at 95°C, annealing for 2 min at 58°C, extension for 2mins at 72°C, with a final extension for 10 min at 72°C were used. For *amoA*, annealing was modified to 51°C.

Shotgun library construction and Sanger sequencing

A small insert library of the metagenome was constructed using approximately 1μg of metagenomic rice endophyte DNA. Briefly, the DNA was randomly sheared to 2-4 Kbp fragments using a HydroShear (GeneMachines) and the sheared DNA purified using AMPure SRPI beads (Agencourt) according to the manufacturer's instructions. The

metagenomic DNA was then end-repaired using the End-itTM DNA End-Repair kit (Epicentre), followed by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation. Approximately 20 ng of DNA was then blunt-end ligated into 100 ng of pUC19 vector overnight at 16ºC using T4 DNA ligase (Roche Applied Science) and 10 % (vol/vol) polyethylene glycol (Sigma). After phenol-chloroform extraction and ethanol precipitation, 1 µl of ligation product was electroporated into ElectroMAX DH10B™ Cells (Invitrogen) and clones prepared and sequenced on an ABI PRISM 3730 capillary DNA sequencer (Applied Biosystems) according to the JGI standard protocols (www.jgi.doe.gov). End-sequencing yielded 175,872 reads totalling 108.29 Mbp of Sanger sequence.

For phylogenetic inference of Bacteria and Archaea, 16S rRNA gene sequences were analysed using the ARB phylogenetic software package (Ludwig et al., 2004), against a representative subset of an internally curated 16S rRNA gene database.

Metagenome assembly

The sequence reads were vector and quality trimmed with lucy version 1.19p (Chou & Holmes, 2001), resulting in 138,989 reads, which were screened for contamination with rice plant DNA using megablast against the *Oryza sativa* genome (>=98%id, min e-30). After removal of 59,331 rice sequences, the resulting 79,658 host plant-free metagenomic endophyte reads were assembled using the Paracel Genome Assembler 2.6.2 (PGA). The assembly size comprised 14,739,389 bp in 10,583 contigs, 236 of which were major contigs defined as being at least 10 reads deep and 2 Kbp long. The N50 contig length was 1420 bp and the longest contig was 13.4 Kbp.

To estimate the effective genome size, the method of Raes et al. (2007) was applied; in brief, it assesses the number of 35 selected marker genes that are generally single copy genes, thus their density propriety is inversely linear to a representative microbial genome size prevalent in the metagenome analyzed.

Gene prediction and annotation

The metagenomic assembly was loaded into IMG/M-ER (Expert Review for Metagenomes) for gene predictions and annotation as described in the "Standard Operating Procedure for the Annotations of Genomes and Metagenomes submitted to the IMG-ER System" document (http://img.jgi.doe.gov/er/doc/about_index.html). The sequence data were deposited in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) under project accession ADIE01000000.

Comparative metagenome analysis

Abundances of specific protein-encoding genes were compared for selected metagenomes obtained by the same sequencing method (NCBI project numbers: Diversa sileage (Minnesota soil), 13699, 152 Mbp; Australian sludge Phrap assembly, 17659, 53 Mbp; US sludge phrap assembly, 17657 56.6 Mbp; Termite gut, 19107, 71 Mbp; human gut subject 6 and 7 pooled, 16729, 33 Mbp; O. algarvensis endosymbionts Delta 1, Delta 4, gamma 1, gamma 3 pooled, 17779, 13.5 Mbp; Whalefall samples 1, 2, 3 pooled, 13700, 75 Mbp). Gene counts were normalized per Mb of metagenome sequence. Proteins were routinely identified by domains for specific functions (pfam or TIGRFam), or by COG category, or in some cases (quorum sensing systems, nitrogenase, ACC deaminase, IAA production etc.) by sequence similarity searches (BLASTP, e-value < 1e-5) in comparison to well-studied proteins.

Results and Discussion

Metagenome characteristics

Endophytic bacterial cells inside plant tissues, tightly attached to host cells, are difficult to extract and separate from plant cells and contaminating surface bacteria. We therefore first established successfully a protocol for endophyte cell extraction and enrichment from roots, using flowering *Oryza sativa* cv. APO plants grown on IRRI experimental fields. Mechanical removal of rhizoplane populations had been demonstrated previously, using vigorous shaking with glass beads (Reinhold et al., 1986). As the culturable endorizosphere population was found to be entirely different from the rhizoplane population (Reinhold et al., 1986), this treatment appeared to be efficient. The stepwise removal of non-endophytic bacteria from roots and the stability of the endophyte consortium was for our experiment confirmed by *nifH*-DNA-based community analysis (T-RFLP) of N_2-fixing bacteria in samples from different stages of the procedure (Fig. 27). The *nifH* gene profiles obtained from total roots were altered by surface treatment: the relative abundance of several OTUs decreased. Surface-treated roots harbored OTU profiles similar to those of endophyte cells extracted from the same root pool (plot 1), indicating that endophyte extraction was successful and endophytes had been enriched.

Small-insert shotgun libraries were generated from DNA extracted from the endophyte community, and Sanger sequencing yielded approximately 47 Mb of bacterial DNA sequences. The average endophyte genome size based on typical single-copy genes (Raes

et al., 2007) was calculated as 5.7 Mbp. Out of more than 64,000 protein-encoding genes, 36% of the annotated genes could be categorized into a COG category and 37% encoded defined pfam domains.

Community composition

The analysis presented here is the as yet most comprehensive survey of the taxonomic affiliation of uncultured endophytes and revealed a rather limited diversity in comparison to the microbial diversity encountered in soil. Phylogenetic distribution of endophytes, based on rRNA genes in the metagenome library and thus not biased by PCR amplification, corresponded very well with the taxonomic distribution of protein-coding genes (Fig. 28). *Proteobacteria* dominated the endophyte community, with *Gammaproteobacteria* and *Alphaproteobacteria* related to *Enterobacter* and *Rhizobia* being highly abundant (Fig. 23 and 28). They are thus likely to contribute the predominant genomes covered by our functional analysis below. Within the *Enterobacteriaceae*, predominant sequences were related to an endophyte strain previously isolated from rice (*Enterobacter* sp. CBMB30) (Lee et al., 2006). In addition to Firmicutes, few members of other phylogenetic lineages were detected that typically occur in soil but were not expected inside plants (*Verrumicrobiae, Planctomycetes* and *Fusobacteria*). Thus the taxonomic range of putative endophytes was extended by our study. Other common soil prokarya such as *Acidobacteria* or *Archaea* were absent, despite methanogens being generally important members of the rice rhizosphere community (Lu & Conrad, 2005; Shrestha et al., 2009). Rice roots that develop aerenchyma provide a source of oxygen in an otherwise anoxic flooded soil. Therefore, oxygen may deter methanogens from colonizing the rice root interior. In contrast, the detection of various genes required for methane oxidation in the metagenome including *pmo*, *mmo* and *mxa* genes, evidenced the presence of endophytic methane-oxidizing bacteria. The predominant endophyte community was thus quite different from the consortia that are typically encountered in rice paddy soil.

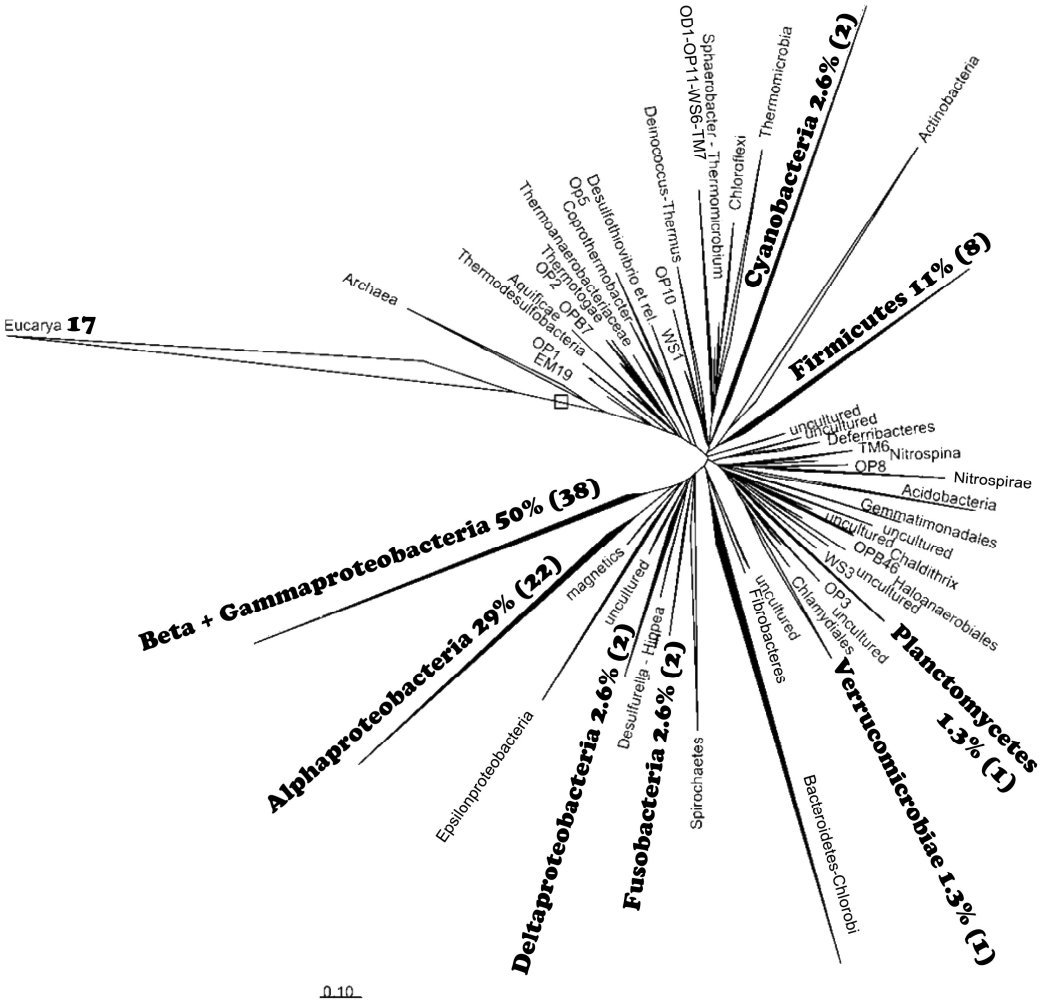

Fig. 23 Phylogenetic analysis of rice endophyte metagenome

Neighbor-joining phylogenetic tree of prokaryotic and eukaryotic 16S/18S rRNA sequences showing the phylogenetic distribution of microbial endophytes detected in the metagenome sequences (in bold Phyla/Classes). Sequences were aligned and the tree was constructed using the ARB phylogenetic software package. The bar indicates 10% evolutionary distance. About one third of the 16S rRNA genes found in the metagenomic library showed less than 97% identity with sequences deposited in public databases. In parenthesis numbers of clones, percentage given for prokaryotic sequences.

Establishment of endophytes in the host

Sanger sequencing yielding sufficiently large sequence tags permitted us to predict more than 64,000 protein-coding genes. Annotation based on protein sequence identities and protein domain analyses allowed us to identify metabolic capacities, potentials and features presumably important for interaction with the plant. In addition, comparison of the relative abundances of protein-coding genes in the endophyte and selected soil, sludge, gut and other metagenomes (see Methods) allowed us to identify processes that are likely important for the endophyte community, providing novel targets for future research e.g. on interaction mechanisms. All the metagenomes used for comparison were based on the

same sequencing technology, and of a similar size range (13 – 152 Mbp, with the rather diverse soil metagenome as largest one). With respect to many of the characteristics abundantly detected in the endophyte metagenome (see below), gene abundances in metagenomes of soil and sludge samples were lower and rather similar to each other.

Several sets of protein-coding genes were abundant in the endophyte metagenome, which allows ascribing numerous capacities to this community. Some of these features might reflect adaptations of the bacterial community to the endorhizosphere as an exclusive microhabitat.

The outcome of plant-bacterium interactions is often determined by bacterial protein secretion systems (Downie, 2010). In the endophyte metagenome, all known protein secretion systems for translocation across the cytoplasmic and outer membranes were present (Fig. 30, 31 and 32). An exception was the T3SS commonly used by symbiotic (Downie, 2010) and pathogenic bacteria to inject effector proteins directly into the host cytoplasm and thereby to modulate the host response. New targets for functional studies are proteins whose role in interactions has not yet been studied in cultivated endophytes: high representation of genes encoding components of T6SS, suspected to deliver effector proteins into cells of eukaryotic hosts (Pukatzki et al., 2009), suggests their importance in beneficial plant-microbe interactions. Interestingly this distribution is also reflected in genomes of cultivated endophytic bacteria, which harbor e.g. at least one (*Klebsiella pneumoniae* 342 (Fouts et al., 2008), *Enterobacter* sp. 638 (Taghavi et al., 2010)) or two (*Azoarcus* sp. BH72 (Krause et al., 2006)) putative type VI secretion gene clusters. Striking was also the high relative abundance of domains typical for autotransporters and the chaperone/usher pathway.

Hydrolytic, plant polymer-degrading enzymes were identified as another putative important feature. Their high gene abundance and diversity were only exceeded by that found in the termite gut community (Fig. 33), a microbial community specialized towards plant lignocellulose degradation (Warnecke et al., 2007). Especially pectinases prominent in the metagenome may contribute to endophyte entry into and spreading inside roots by degrading middle lamella. The role of hydrolytic enzyme has e.g. already been shown for the endoglucanase EglA in the endophyte *Azoarcus* sp. BH72 involved in rice root invasion (Reinhold-Hurek et al., 2006).

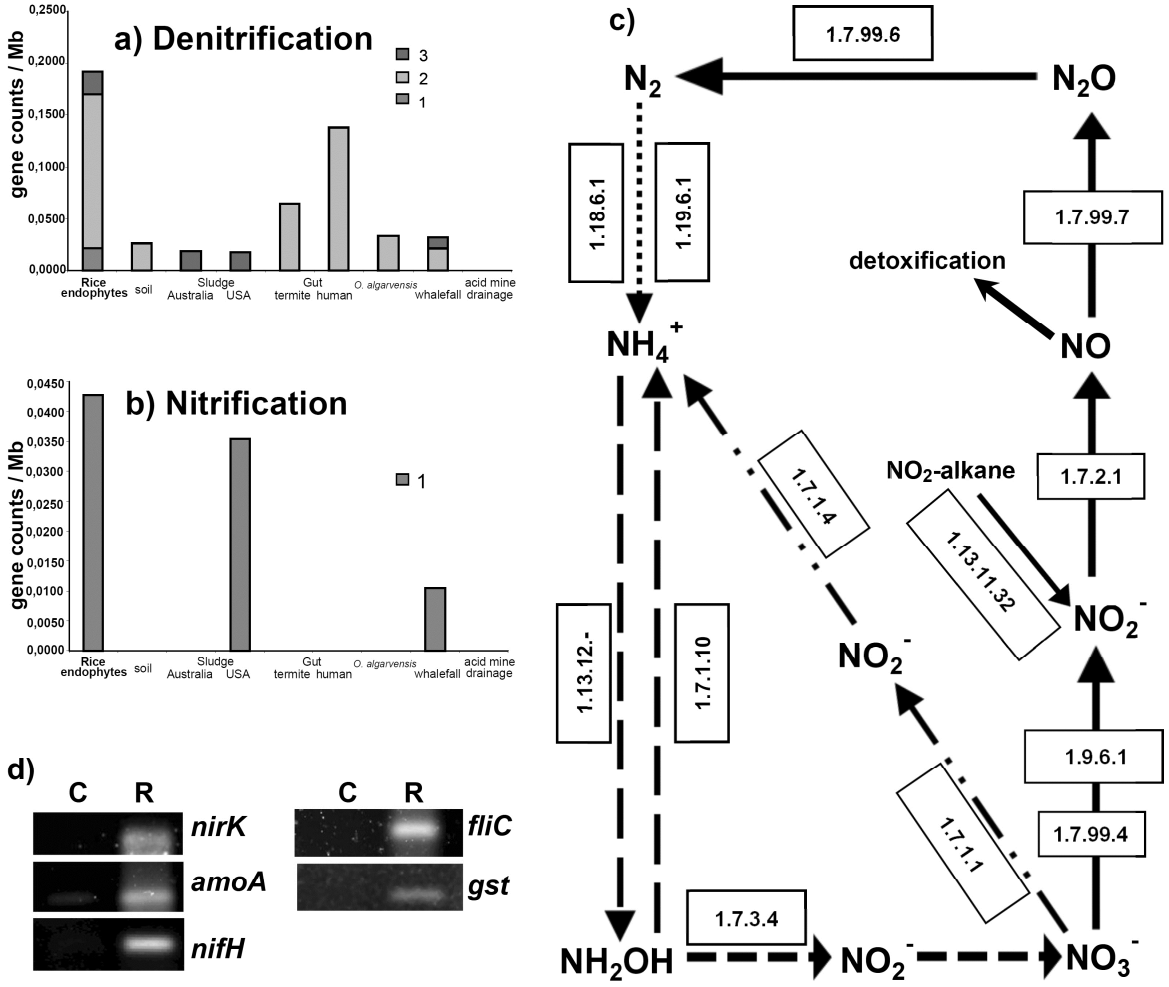

Fig. 24 Nitrogen cycling based on rice endosphere metabiome analysis

Pathways of nitrogen metabolism represented in the endophytic metagenome of rice, adapted with modification from Kyoto Encyclopedia of Genes and Genomes (KEGG) (**c**). The nitrogen fixation (dotted arrow), denitrification (full arrows), nitrification (dashed arrows) and nitrate assimilation (dashed-dotted arrows) are shown. The numbers inside boxes depict the EC number of the enzymatic reaction for which protein(s) were detected in the endophytic metagenome. Upper left panels, comparison of abundance of genes coding for proteins putatively involved in denitrification (**a**) or nitrification (**b**) in different metagenomes, normalized as gene counts per Mb. Gray code for protein functions is in (**a**) 1, periplasmic nitrate reductase NapE proteins (pfam06796); 2, formate/nitrite transporters (pfam00324); 3, one of the accessory proteins of the *nos* (nitrous oxide reductase) gene cluster (pfam00496): in (**b**) 1, putative ammonia monooxygenase (pfam05145). (**d**) Transcripts of selected gene fragments in RNA extracts of rice roots cv. Apo related to the N-cycle (left) and other features (right), as detected by RT-PCR. C, control for DNA contamination (heat-inactivated reverse transcriptase); R, reaction with reverse transcription. *nirK*, encoding nitrite reductase related to *Bradyrhizobium*, 416 bp; *amoA*, encoding gammaproteobacterial ammonium monooxygenase, 270 bp; *nifH,* encoding iron protein of nitrogenases, 362 bp; *fliC*, encoding gammaproteobacterial flagellin, 141 bp; *gst*, encoding *Bradyrhizobium*-related GST, 327 bp.

Although flagellins are known to elicit an innate immune response in Arabidopsis (Zipfel et al., 2004), all components of the flagellar apparatus were encoded in the rice endophyte metagenome. As they were more abundant than in other metagenomes except for the termite gut flora (Fig. 32), motility or flagella-mediated adhesion may be required

for establishment in the rice endosphere in comparison to e.g. soil. This is also supported by detection of transcription of flagellin-encoding endophytic *fliC* in rice roots (Fig. 24).

The rice endophyte metagenome contained an extremely high number and diversity of genes encoding enzymes potentially involved in the detoxification of ROS, as well as glutathione synthases and also GST (Fig. 34, Table 8), that was transcribed in association with roots (Fig. 24). As plants produce a range of ROS in response to abiotic stress or to colonizing microorganisms which elicit an oxidative burst, the abundance in the metagenome and in cultivated plant-associated bacteria (Fouts et al., 2008; Taghavi et al., 2010) suggests that endophytes require these enzymes to be able to successfully colonize plants.

Although in an anoxic paddy soil iron should not be limiting, the rice root itself obtains some oxygen through its aerenchyma, and therefore the bioavailability of iron should be reduced. Indeed our gene analysis indicates that the root interior appears to be a microenvironment extremely depleted of bioavailable iron: The endophyte metagenome showed a strikingly high number of genes encoding proteins that are potentially involved in siderophore biosynthesis (compounds of low molecular mass with high specific affinity towards ferric iron or other metal ions, typically secreted to the extracellular milieu) (Fig. 35). In accordance, siderophore receptors, the so-called TonB-dependent receptors required for the uptake of ferric-siderophore complexes, and other elements for the uptake of iron as well as iron storage proteins were also found in high abundance (Fig. 35). Also in genomes of all cultivated and sequenced endophytes, these genes were abundantly present (e.g. *Azoarcus* sp. BH72 (Krause et al., 2006), *Klebsiella pneumoniae* 342 (Fouts et al., 2008), *Enterobacter* sp. 638 (Taghavi et al., 2010)). This also suggests a high potential of the rice endophyte community to compete for iron with other members of the microbial community and perhaps with the plant host.

The endophyte microbiome comprised high diversity (more than 80% of the known pfam domains were detected) and abundance of transcriptional regulators, only exceeded by the human gut metagenome (Fig. 40). A subset was highly represented, such as the LysR-, Crp- and the IclR-families of transcriptional regulators, which are relevant for bacterial virulence, quorum sensing and metabolic adaptation. This suggests a very high degree of plasticity of responses to varying environmental stimuli - such as plant signals -, although the endosphere microenvironment was previously thought to be a rather stable, protected environment (Krause et al., 2006).

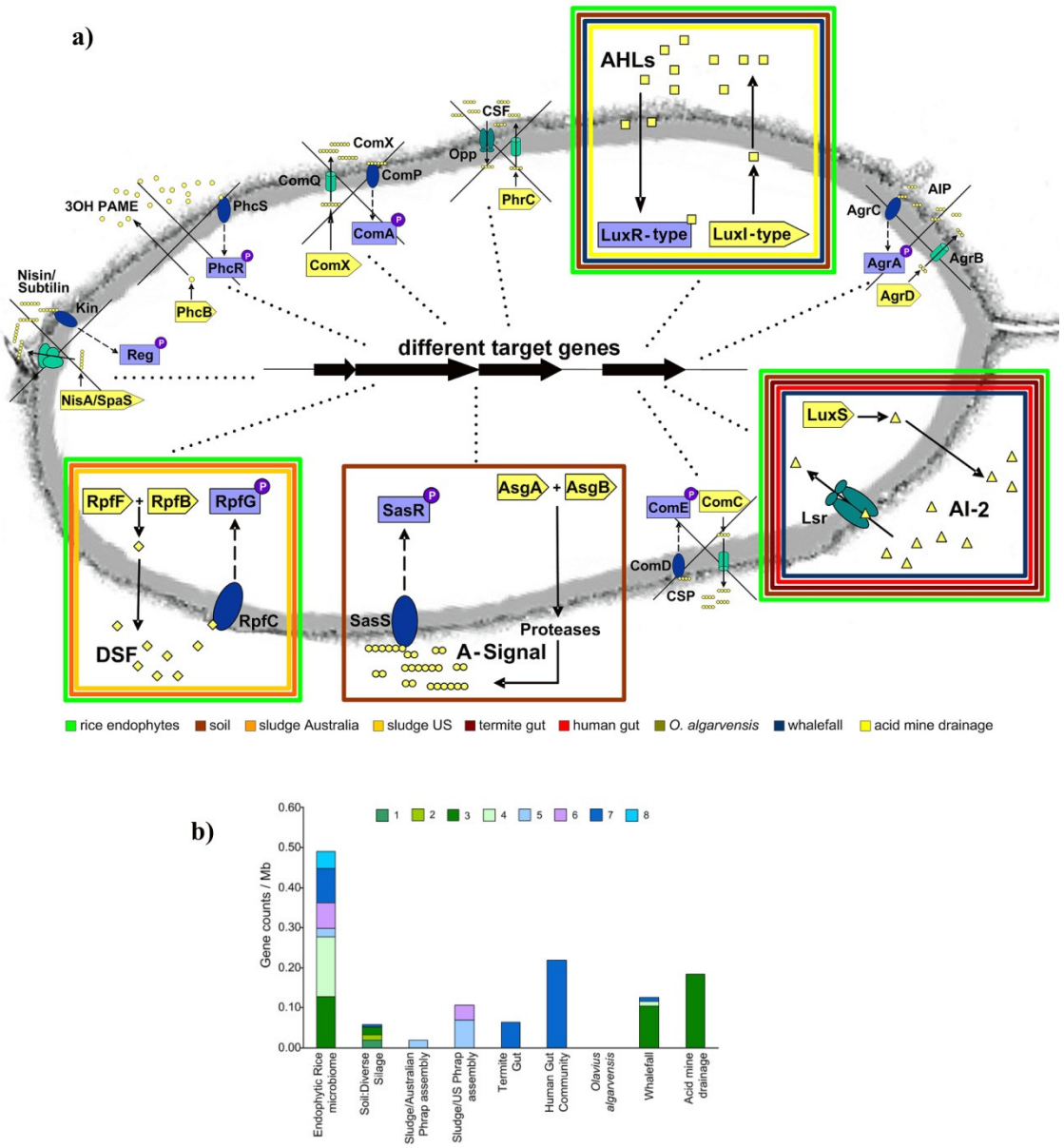

Fig. 25 QS systems detected in metagenomes

Quorum sensing (QS) systems detected in metagenomes by domain and sequence similarity analyses. **(a)** Proteins for signal molecule synthesis and detection that were encoded in the analyzed metagenomes are boxed in the respective colors (see legend below). Known QS systems for which no evidence was found in any of the tested metagenomes are crossed out. QS systems are labeled as AHL, AI-2, DSF, A-signal system, CSF (sporulation factor system), ComX system, CSP (competence stimulating peptide system), AIP (autoinducing peptide system), 3-OH PAME system and Nisin/Subtilisin system. Proteins that are involved in signal molecule synthesis (yellow arrows), detection (blue ellipses and blue boxes) and transport (green ellipses and barrels) are indicated with the particular protein name. (**b**) Comparison of abundance of genes coding for QS signal molecule synthesis and detection in different metagenomes, normalized as gene counts per Mb. Colour code for protein functions is 1, A-Signal-synthesis (AsgA/AsgB); 2, A-Signal-detection (SasS/SasR); 3, AHL-synthase (LuxI-type); 4, AHL-detection (LuxR-type); 5, DSF-synthesis (RpfB/RpfF); 6, DSF-detection (RpfC/RpfG); 7, Autoinducer-2 synthase (LuxS); 8, Autoinducer-2-detection (Lsr).

Microbial communication by autoinducer molecules might be highly important for the rice endophytic community. Three systems (AI-2, DSF and AHL) were identified in the

endophytic rice microbiome. The AI-2 system (which has been commonly found in metagenomes) is known for both Gram-negative and Gram-positive bacteria, where it acts as a global signal molecule for interspecies communication (Williams et al., 2007). All LuxS sequences found in the rice endophytes and whalefall metagenomes were gammaproteobacterial, whereas the metagenome samples from soil, human and termite guts contained only sequences from Gram-positives like the *Actinobacteria* and *Firmicutes*. Interestingly, the DNA-binding transcriptional activator SdiA was detected in the rice endophyte metagenome. In *Escherichia coli* and *Salmonella enterica* SdiA appears to encode a receptor that exclusively detects signal molecules of other species (Michael et al., 2001), indicating the importance of quorum sensing molecules and their receptors in a diverse bacterial community. Thus, genes encoding proteins for autoinducer synthesis as well as for detection were most abundant in the endophyte metagenome (Fig. 25), where three different autoinducer systems were identified (Table 9) - a high diversity in comparison to other metagenomes. This probably reflects a need for concerted gene regulation for virulence and colonization, even across species borders.

Metabolic adaptations

Our gene analysis suggested numerous metabolic adaptations of endophytes to their microenvironment. Partially they might relate to a gradient of oxygen concentrations between anoxic soil, microaerobic aerenchymatic tissues and O_2 depletion in bacterial microcolonies. In addition to fermentative abilities (Fig. 36, Table 10), which might be required to cope with anoxic niches, the abundance of alcohol dehydrogenases indicated that ethanol accumulated by rice roots at low O_2 concentrations might be an important carbon source, as suspected for the grass endophyte *Azoarcus* sp. strain BH72 (Krause et al., 2006). The high abundance of genes for transport systems, mainly ABC family transporters for several amino acids or polyamines (Fig. 37) indicated that they may be important N- or C-sources derived from the plant. The high abundance of genes involved in the degradation of aliphatic and aromatic compounds in comparison with other selected metagenomes (Fig. 38, Table 8) suggested that the rice endophyte community is well equipped to degrade a wide range of aromatic compounds derived from the plants' secondary metabolism, or pesticides and herbicides applied in agriculture. Overrepresented genes encoding proteins for the synthesis and degradation of carbon storage compounds such as polyhydroxyalkanoates (PHA) or polyhydroxybutyrate (PHB) (Fig. 39, Table 8) may enhance survival during starvation and tolerance to stress (Kadouri et al., 2003), but

also PHB may serve as a redox regulator for the removal of growth inhibitory metabolites (Aneja et al., 2004) such as plant secondary metabolites.

Plant growth promotion and biocontrol

Our analysis also revealed features of endophytes that are likely to affect plant yield. We detected numerous traits in the endophyte metabiome that are likely to shape the fitness and performance of their host, and might therefore be exploited for improvement of agricultural management practices. Plant growth may be promoted, and stress tolerance and nutrient uptake improved by providing phytohormones such as IAA (Spaepen et al., 2007) or CKs (Timmusk et al., 1999) and by producing the enzyme ACC deaminase (Glick, 2004). All corresponding genes were detected in the endophyte metagenome, for IAA production even for the three pathways: the IAM, the IPyA and the tryptamine pathway (Table 9). Also, numerous antioxidant activities (see above) might foster stress tolerance of the host plant.

Although degradative capacities for aromatic compounds are commonly found in soil bacteria, the endophyte metagenome surprisingly harbored a far higher number of genes for ring-cleaving dioxygenases. This emphasizes the potential of endophytes for bioremediation. As iron and metal transport and storage mechanisms were strikingly highly represented (see above), endophyte-plant systems might also be developed to affect heavy metal transport and phytoremediation.

With respect to plant health, the pronounced gene abundance for siderophore production indicates strong biocontrol capacities allowing to compete with pathogens for iron, as suspected for some cultured endophytes (Krause et al., 2006; van der Lelie et al., 2009). Also, bacterial QS autoinducers may participate in the interaction of plants with pathogens and symbionts by induction of plant gene expression (Mathesius et al., 2003), e.g. conferring systemic resistance to pathogens (Schuhegger et al., 2006). The high diversity of autoinducer molecules represented in the endophyte microbiome (see above) and the possibility of novel autoinducers (Krause et al., 2006) advocate a strong, underexplored impact of endophytes.

Nitrogen cycle

Nitrogen is the nutrient most limiting to plant productivity in terrestrial ecosystems and in rice production. Losses of N-fertilizer depend on nitrogen use efficiency (NUE), which can be affected by microbial N-cycling that is mainly thought to occur in soil (Li et al., 2008). Surprisingly, microbial nitrogen cycling was almost completely represented in the

endophyte metagenome, although different steps require different oxygen concentrations: we found gene-based evidence for aerobic (nitrification), microaerobic (nitrogen fixation) and anaerobic (denitrification) parts of the N-cycle which were highly overrepresented in comparison to soil (Fig. 24 and Table 8). Among several domains of enzymes typical for denitrification, the NapE domain (pfam06796, periplasmic nitrate reductase) appeared to be unique for the endophyte metagenome. Furthermore, domains for formate and nitrate transporters and NosL (nitrous oxide reductase) were also abundant. Additionally, components of the assimilatory nitrate reductase pathway to assimilate ammonium from nitrate were well represented. AmoA (pfam05145), which can be considered to specifically represent putative bacterial ammonia monooxygenases for nitrification, was highly enriched in the endophyte metabiome. Transcript detection by RT-PCR suggested even concurrent root-associated microbial activity in the three major branches of the N-cycle including nitrogen fixation (Fig. 24). Nitrification and bacterial ammonia oxidation in the rhizosphere were thought to be major contributors to rice nutrition (Li et al., 2008), however our data suggest that also the rice root endophytes may impact growth and nitrogen use efficiency in rice. Their activities should particularly be taken into account when consequences of shifting flood regimes to aerobic rice cultivation are assessed.

Remarkable was the high apparent density (Fig. 24) of diazotrophic endophytes suggesting an N-poor microenvironment: amongst an average eight sequenced genomes, five *nifH* genes were detected, indicating that more than 50% of the endophytes were diazotrophic. They were predominantly related to uncultured *Bradyrhizobium* - *Xanthobacter* strains (Fig. 29 and Table 8). Despite moderate N-fertilizer application, root-associated *nifH* transcription was active (Fig. 24), which underscores the role of BNF in this habitat. Phylogenetic analysis of *nifH* clone libraries prepared from DNA and mRNA of total roots showed that the entire community was rather diverse, comprising also *Geobacter* spp. typically occurring in soil. However only a minority of root-associated diazotrophs was active in BNF (Fig. 29). Activity was mostly located in the alphaproteobacterial clade related to *Rhizobium*, which extends the typically root-nodule associated rhizobial nitrogen fixation activity to cereals.

Conclusion

Our metagenome analysis revealed insights beyond the genome information of individual bacterial strains. Bacterial endophyte communities seem to be highly adapted to proliferate and spread within plants (for an overview see Fig. 26). The observed differences Rice and

also other plants may be considered to be rich reservoirs of bacterial activities affecting plant growth and health. A deeper understanding of endophyte functions and mechanisms for their establishment in the endosphere could be exploited to improve agricultural management practices with respect to biocontrol, bioremediation, and plant nutrition. A direct comparison of endophyte, rhizosphere soil and soil metagenomes from the same site would further advance our knowledge of microbial adaptations.

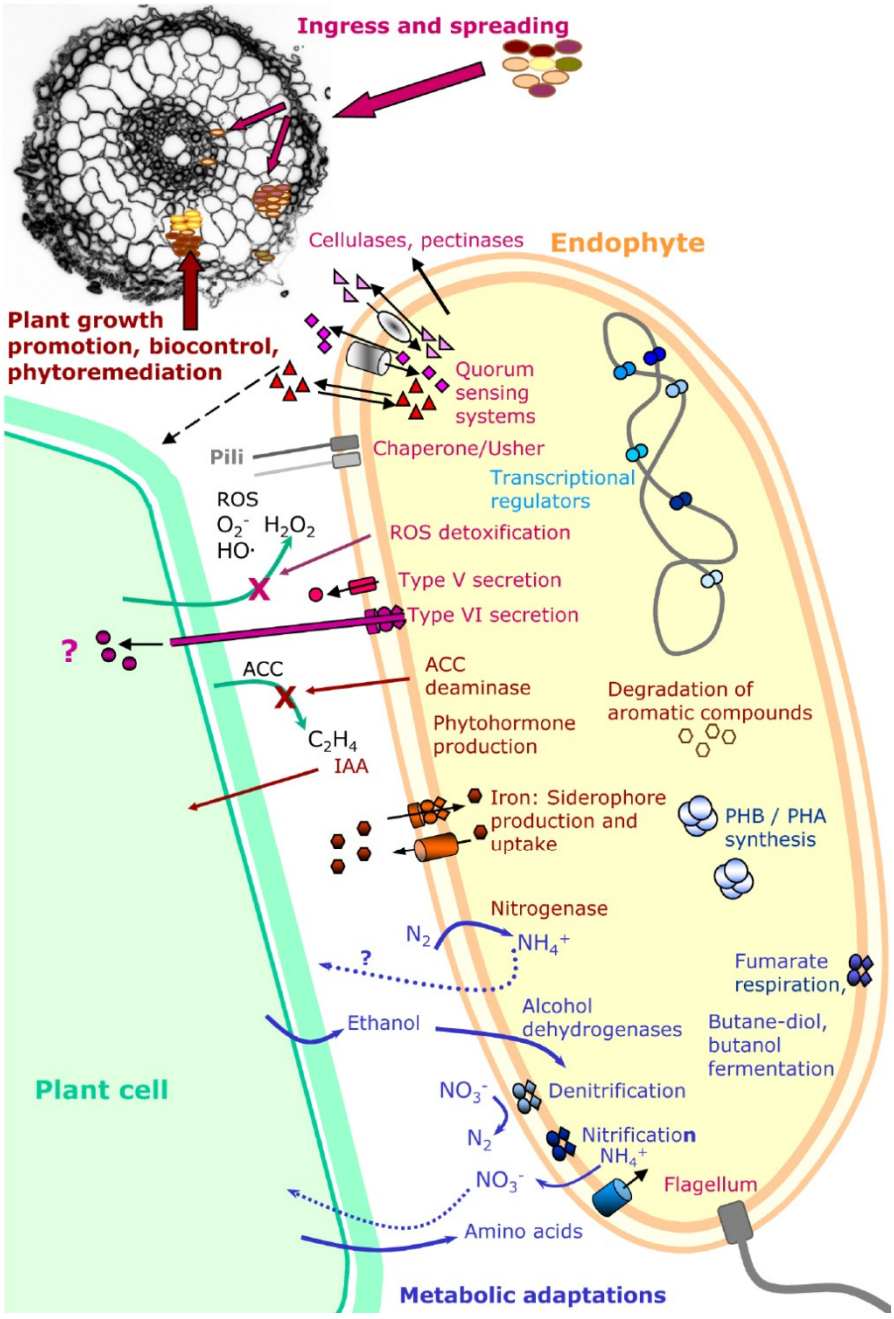

Fig. 26 Overall functions of rice endohpytes

Reconstruction of rice – endophyte interactions inferred from quantitative gene content analyses of the rice endophyte metagenome in comparison to reference metagenomes. The endophyte community is depicted as one cell for reasons of simplicity, however it should not be inferred that all processes are active in one cell simultaneously. In red, processes putatively related to ingress into roots, spreading and establishment

within roots; in brown, processes putatively related to plant growth promotion, biocontrol and phytoremediation; in blue, putative metabolic adaptations. Question mark (?), unknown effector proteins of protein secretion system and unknown transfer of fixed nitrogen to the plant.

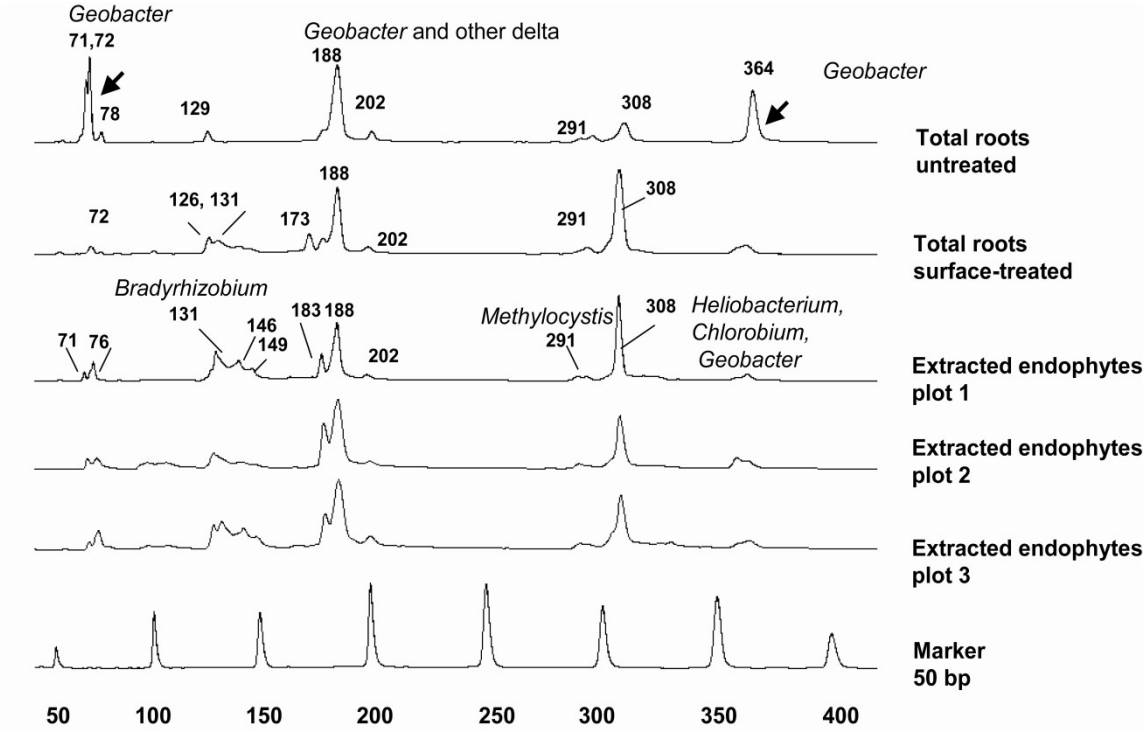

Fig. 27 Extraction of rice endosphere DNA efficiency

nifH-gene profiles associated with roots of *O. sativa* cv. Apo (IR55423-01) and of extracted endophyte cells. Total DNA was extracted from untreated washed roots, from roots treated for removal of the rhizoplane population, from endophyte cells extracted from the same root pool (plot 1) and from endophyte cells of root pools from two different plots harvested within 2 weeks (plots 2 and 3). T-RFLP patterns were obtained from PCR-amplified *nifH*-fragments by restriction endonuclease digestion with *Bst*UI (*Bsh*12361, Fermentas). Numbers indicate sizes of the restriction fragments. For fragments corresponding to *in silico* restriction fragments from *nifH* clone libaries, most closely related cultivated diazotrophs are indicated. Arrows indicate OTUs with reduced abundance after root surface treatment. Similar results were obtained for three replicate DNA extractions.

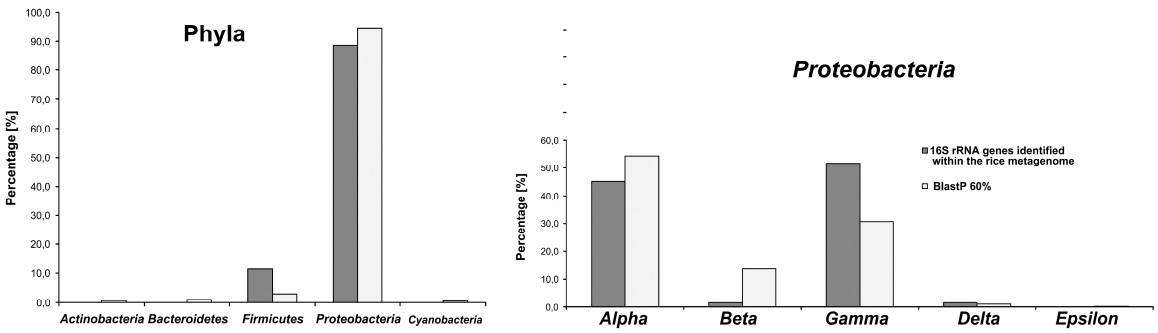

Fig. 28 Phylogenetic distribution of rice endophyte metabiome

Phylogenetic distribution of rice endophytes at the level of selected phyla and class level (*Proteobacteria*) revealed by 16S rRNA genes identified in the rice endophyte metagenome and on best BlastP hits with a cutoff value of 60% homology.

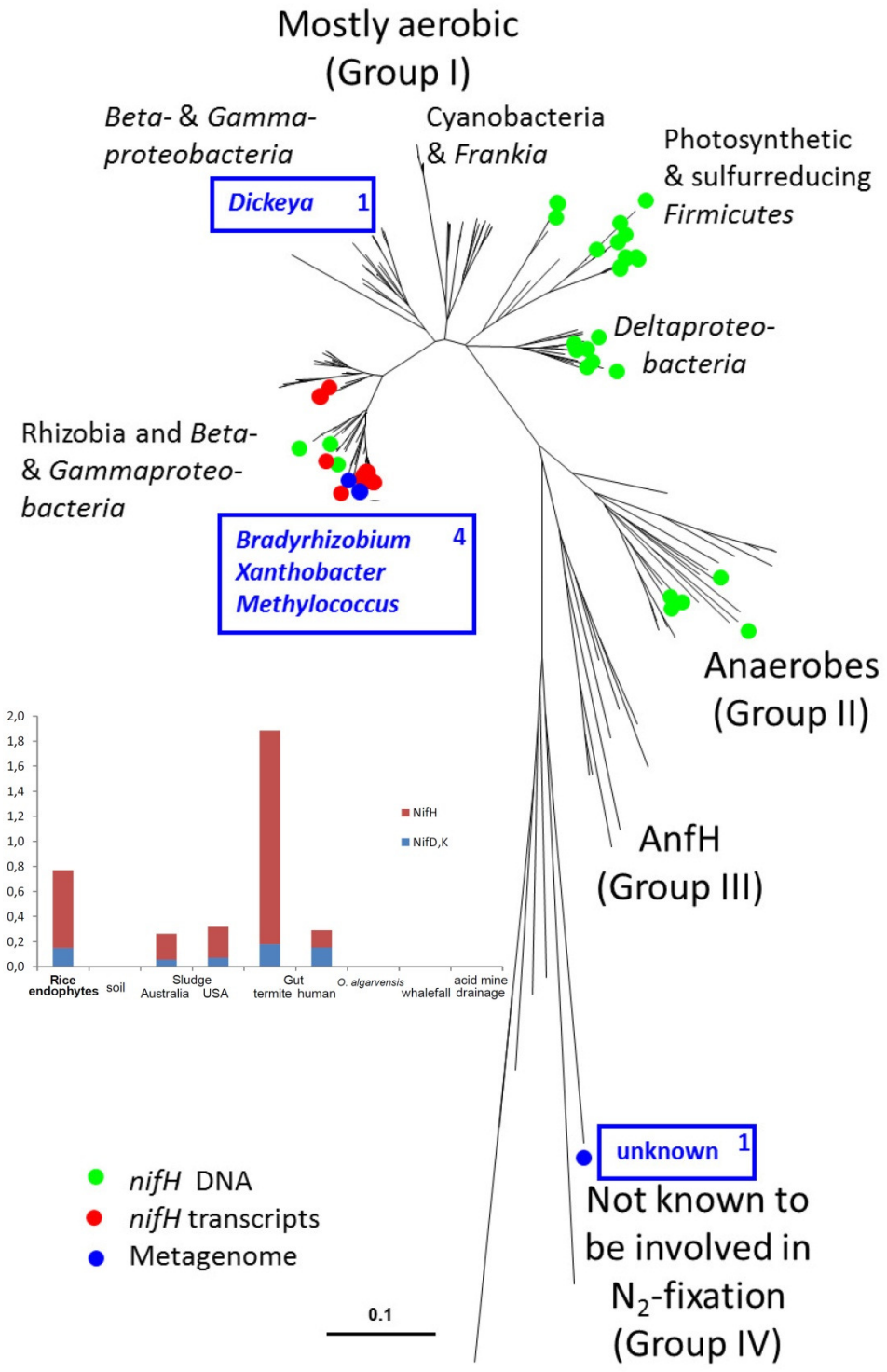

Fig. 29 Phylogenetic affiliation of *nifH* fragments recovered from roots of rice
Minimum-evolution NifH protein tree showing the phylogenetic affiliation of *nifH* fragments recovered from roots of an *O. sativa* cv. Apo plant (plot 2). Fragments of *nifH* were amplified by PCR or RT-PCR, respectively, from DNA (green dots) or RNA (red dots) extracted from untreated washed roots. NifH sequences retrieved from the endophyte metagenome library – not all of which overlapped with the PCR-generated sequences and were mapped on the tree by BLAST analysis - are labeled in blue, numbers of clones are indicated. Clusters are named according to typical cultivated representatives. Insert: Comparison of abundance of pfam domains of nitrogenase proteins encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. 1, pfam00142 (Fer4_NifH); 2, pfam00148 (Oxidored_Nitro).

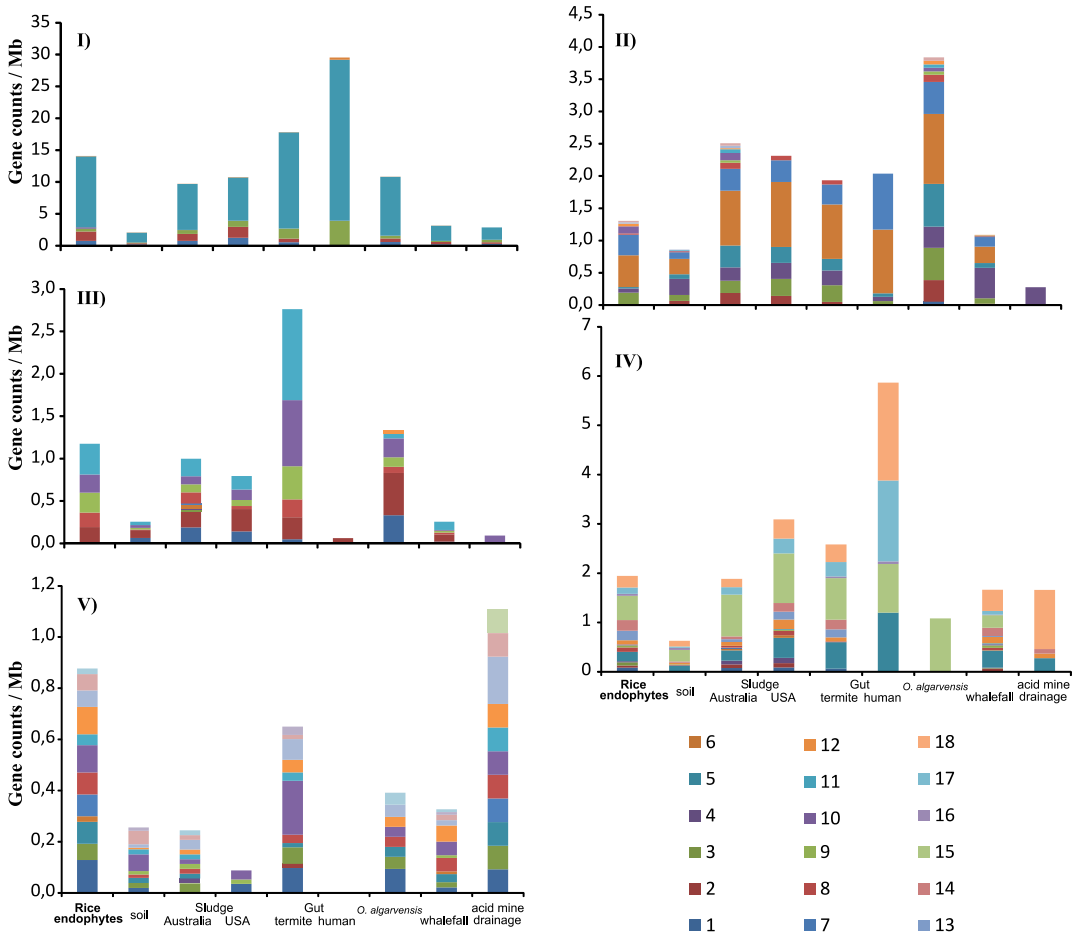

Fig. 30 Distribution of protein secretion system acroos metagenomes

Analysis of protein secretion systems through the entire cell envelope. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. Proteins potentially involved in forming (**I**) Type I secretion systems, (**II**) Type II secretion systems, (**III**) T3SS, (**IV**) Type IV secretion systems, (**V**) T6SS. Designation of domains in (**I**) 1 pfam02321 (OEP), 2 pfam00529 (HlyD), 3 pfam00664 (ABC-membrane), 4 pfam06472 (ABC-membrane_2), 5 pfam00005 (ABC-trans), 6 pfam04392 (ABC_sub_bind); in (**II**) 1 pfam07655 (Secretin_N_2), 2 pfam03958 (Secretin_N), 3 pfam00263 (Secretin), 4 COG1450 (type_II_gspD), 5 pfam05157 (GSPII_E_N), 6 pfam00437 (GSPII_E), 7 pfam00482 (GSPII_F), 8 pfam08334 (GSPII_G), 9 pfam02501 (GSPII_IJ), 10 pfam03934 (GspK), 11 pfam05134 (GspL), 12 pfam04612 (GspM), 13 pfam10741 (GspM_II), 14 pfam01203 (GSPII_N); in (**III**) 1 pfam03958 (Secretin_N), 2 pfam00263 (Secretin), 3 TIGR02500 (YscD/HrpQ family), 4 TIGR02544 (III_secr_YscJ), 5 pfam06188 (HrpE), 6 TIGR02551 (SpaO_YscQ), 7 TIGR01102 (yscR), 8 pfam01313 (Bac_export_3), 9 pfam01311 (Bac_export_1), 10 pfam01312 (Bac_export_2), 11 pfam00771 (FHIPEP), 12 TIGR01399 (hrcV); in (**IV**) 1 pfam04956 (TrbC/VirB2 family), 2 COG3838 (VirB2), 3 pfam05101 (VirB3), 4 pfam03135 (CagE_TrbE_VirB), 5 COG3451 (VirB4), 6 TIGR00929 (VirB4_CagE), 7 pfam07996 (T4SS), 8 pfam04610 (TrbL), 9 COG3704 (VirB6), 10 pfam04335 (VirB8), 11 TIGR02781 (VirB9), 12 COG3504 (VirB9 components), 13 pfam03743 (TrbI), 14 COG2948 (VirB10), 15 pfam00437 (GSPII_E), 16 COG0630 (VirB11 components), 17 pfam02534 (TraG), 18 COG3505 (VirD4); in (**V**) 1 TIGR03344 (VI_effect_Hcp1), 2 TIGR03345 (VI_ClpV1), 3 TIGR03347 (VI_chp_1), 4 TIGR03348 (VI_IcmF), 5 TIGR03349 (IV_VI_DotU), 6 TIGR03350 (type_VI_ompA), 7 TIGR03352 (VI_chp_3), 8 TIGR03353 (VI_chp_4), 9 TIGR03354 (VI_FHA), 10 TIGR03355 (VI_chp_2), 11 TIGR03357 (VI_zyme), 12 TIGR03358 (VI_chp_5), 13 TIGR03359 (VI_chp_6), 14 TIGR03361 (VI_Rhs_Vgr), 15 TIGR03362 (VI_chp_7), 16 TIGR03363 (VI_chp_8), 17 TIGR03373 (VI_minor_4).

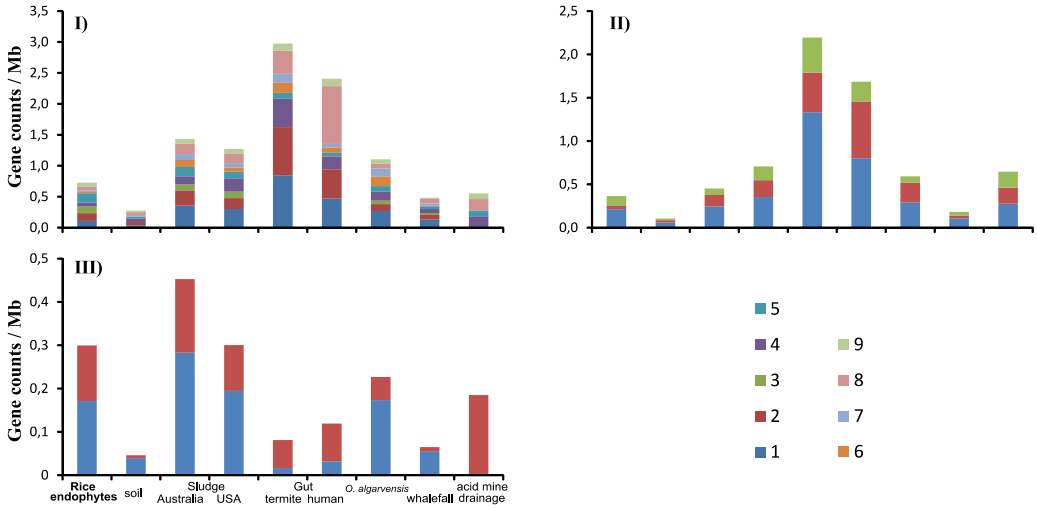

Fig. 31 Distribution of genes involved in the protein secretion systems through the cytoplasma membrane

Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. Proteins potentially involved in forming (**I**) Sec-dependent transport systems, (**II**) signal recognition particle systems, and (**III**) twin-arginine translocation systems. Designation of domains in (**I**) 1 pfam07517 (SecA_DEAD), 2 pfam07516 (SecA_SW), 3 pfam02556 (SecB), 4 pfam02355 (SecD_SecF), 5 pfam07549 (Sec_GG), 6 pfam00584 (SecE), 7 pfam03840 (SecG), 8 pfam00344 (SecY), 9 pfam02699 (YajC); in (**II**) 1 pfam00448 (SRP54), 2 pfam02881 (SRP54_N), 3 pfam02978 (SRP_SPB); in (**III**) 1 pfam02416 (MttA_Hcf106), 2 pfam00902 (TatC).

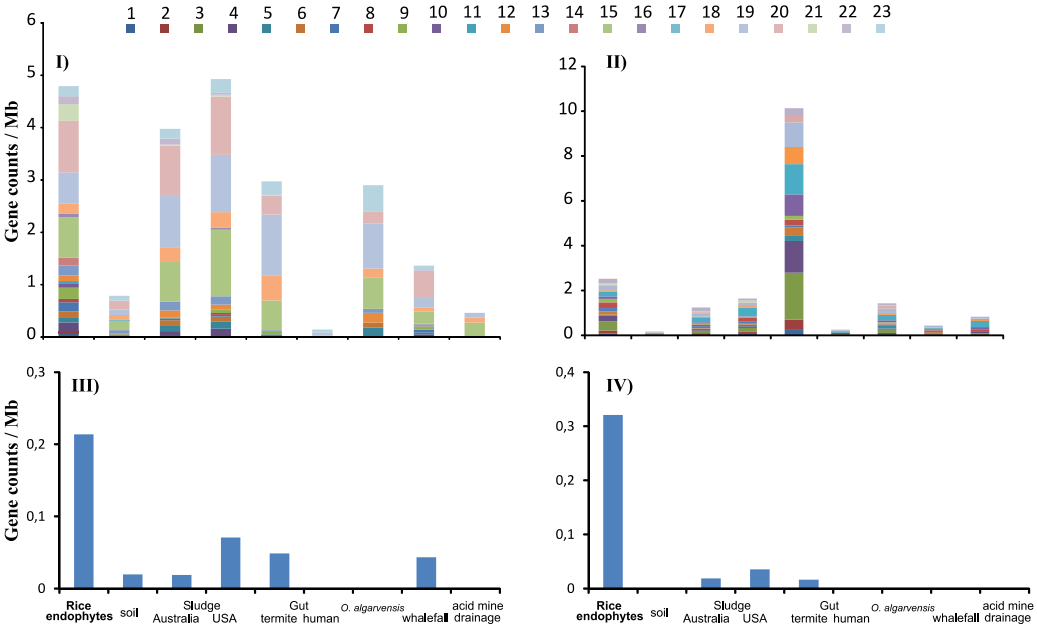

Fig 32 Distribution of selected surface characteristic genes

Analysis of surface characteristics likely to be important for endophytic colonization. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. (**I**) Comparison of pfam domains involved in forming outer membrane channels as listed in TransportDB, a relational database describing predicted membrane channels (http://www.membranetransport.org/). 1, pfam07396 (Porin_O_P); 2, pfam06178 (KdgM); 3, pfam05628 (Borrelia_P13); 4, pfam04966 (OprB); 5, pfam04453 (OstA_C); 6, pfam04355 (SmpA_OmlA); 7, pfam03922 (OmpW); 8, pfam03895 (YadA); 9, pfam03797 (Autotransporter); 10, pfam03573 (OprD); 11, pfam03502 (Channel_Tsx); 12, pfam03349 (Toluene_X); 13, pfam02563 (Poly_export); 14, pfam02530 (Porin subfamily); 15, pfam02321 (OEP); 16, pfam02264 (LamB); 17, pfam01856 (HP_OMP); 18, pfam01103 (Bac_surface_Ag); 19, pfam00691 (OmpA); 20, pfam00593 (TonB_dep_Rec); 21, pfam00577 (Usher); 22, pfam00267 (Porin_1); 23, pfam00263 (Secretin). (**II**) Examination of pfam domains present in proteins forming a flagellar system. 1, pfam02465 (FliD_N); 2, pfam07195 (FliD_C); 3, pfam00669 (Flagellin_N); 4, pfam00700 (Flagellin_C); 5, pfam02120 (Flg_hook); 6, pfam03963 (FlgD); 7, pfam02107 (FlgH); 8, pfam02119 (FlgI); 9, pfam02049 (FliE); 10, pfam01706 (FliG_C); 11, pfam00460 (Flg_bb_rod); 12, pfam02154 (FliM); 13, pfam01052 (SpoA); 14, pfam02108 (FliH); 15, pfam04347 (FliO); 16, pfam00813 (FliP). (**III**) and (**IIV**) Close-up for 9, pfam03797 (Autotransporter), and 21, pfam00577 (Usher), respectively.

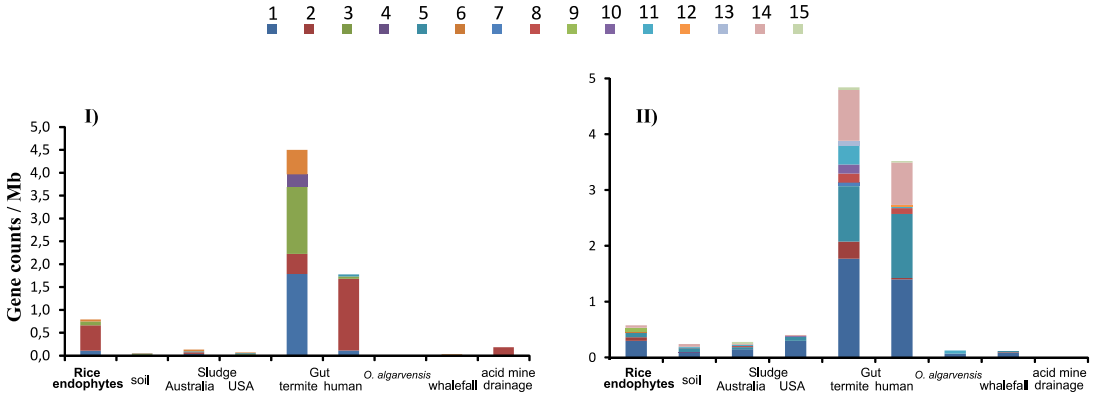

Fig. 33 Distribution of genes involved in the degradation of plant cell wall

Analysis of proteins related to degradation of plant cell wall polymers. Comparison of selected metagenomes for abundance of pfam domains represented in (**I**) cellulases, xylanases, cellobiohydrolases, and cellulose-binding domains, or in (**II**) pectinases, normalized as gene counts per Mb. Color code for protein functions is in (**I**) 1, pfam00150 (Cellulase); 2, pfam00232 (Glyco_hydro_1); 3, pfam00331 (Glyco_hydro_10) 4, pfam00457 (Glyco_hydro_11); 5, pfam00553 (CBM_2); 6, pfam00759 (Glyco_hydro_9) and in (**II**) 1, pfam00933 (Glyco_hydro_3); 2, pfam01270 (Glyco_hydro_8); 3, pfam00942 (CBM_3); 4, pfam01341 (Glyco_hydro_6); 5, pfam01915 (Glyco_hydro_3_C); 6, pfam02011 (Glyco_hydro_48); 7, pfam02015 (Glyco_hydro_45); 8, pfam02018 (CBM_4_9); 9, pfam02839 (CBM_5_12); 10, pfam02927 (CelD_N); 11, pfam03422 (CBM_6); 12, pfam03423 (CBM_25); 13, pfam03425 (CBM_11); 14, pfam04616 (Glyco_hydro_43); 15, pfam06452 (DUF1083).

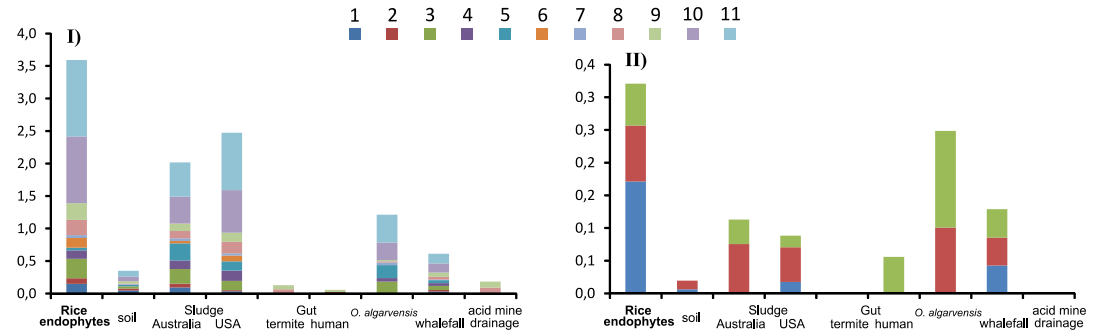

Fig. 34 Distribution of genes involved in defence against oxidative stress

Genes potentially involved in defence against oxidative stress. (a) Comparison of pfam domains potentially involved in detoxification of ROS and products of oxidative stress found in selected sequenced metagenomes. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. Proteins potentially involved (**I**) in Oxygen detoxification: 1 pfam00199 (catalase), 2 pfam06628 (catalase-related immune-responsive), 3 pfam00141 (peroxidase), 4 pfam00255 (GSHPx; glutathione peroxidase), 5 pfam03150 (CCP_MauG; di-haem cytochrome c peroxidase), 6 pfam04261 (dyp-type peroxidase family), 7 pfam00080 (Sod_Cu; copper/zinc superoxide dismutase), 8 pfam00081 (Sod_Fe_N; iron/manganese superoxide dismutases), 9 pfam02777 (Sod_Fe_C; iron/manganese superoxide dismutases), 10 pfam00043 (GST, C-terminal domain), 11 pfam02798 (GST, N-terminal domain). (b) Comparison of pfam domains potentially involved in glutathione synthesis found in selected sequenced metagenomes. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence and (**II**) in Glutathione biosynthesis: 1 pfam02955 (prokaryotic glutathione synthetase, ATP-grasp domain), 2 pfam02951 (prokaryotic glutathione synthetase, N-terminal domain), 3 pfam04262 (glutamate-cysteine ligase).

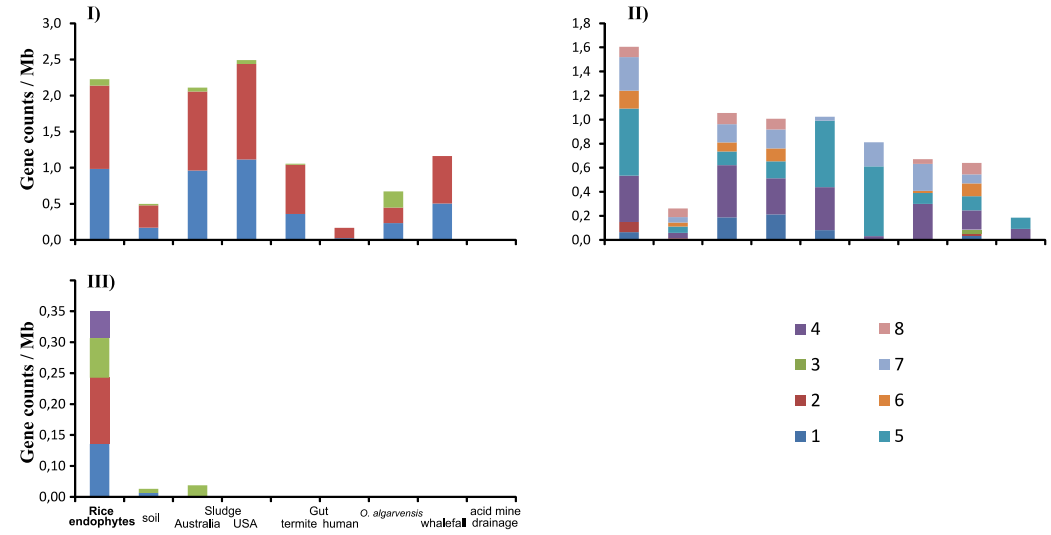

Fig. 35 Distribution of genes involved in iron acquisition and storage

Analysis of proteins for iron acquisition and storage, and production of antibiotics. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. Proteins potentially involved (**I**) in forming TonB-dependent receptors, (**II**) in iron transport and storage, and (**III**) in siderophore production. Some domains in this category may be involved in other processes alternatively. Designation of domains in (**I**) 1, pfam00593 (TonB_dep_Rec); 2, pfam07715 (Plug); 3, pfam07660 (STN); in (**II**) 1, pfam03544 (TonB); 2, TIGR02797 (exbB); 3, TIGR02805 (exbB2); 4, pfam02472 (ExbD); 5, pfam01032 (FecCD); 6, pfam04773 (FecR); 7, pfam01475 (FUR); 8, TIGR00754 (bfr); in (**III**) 1, pfam04183 (lucA_lucC); 2, pfam04954 (SIP); 3, pfam08021 (FAD_binding_9); 4, pfam10331 (AlcB).

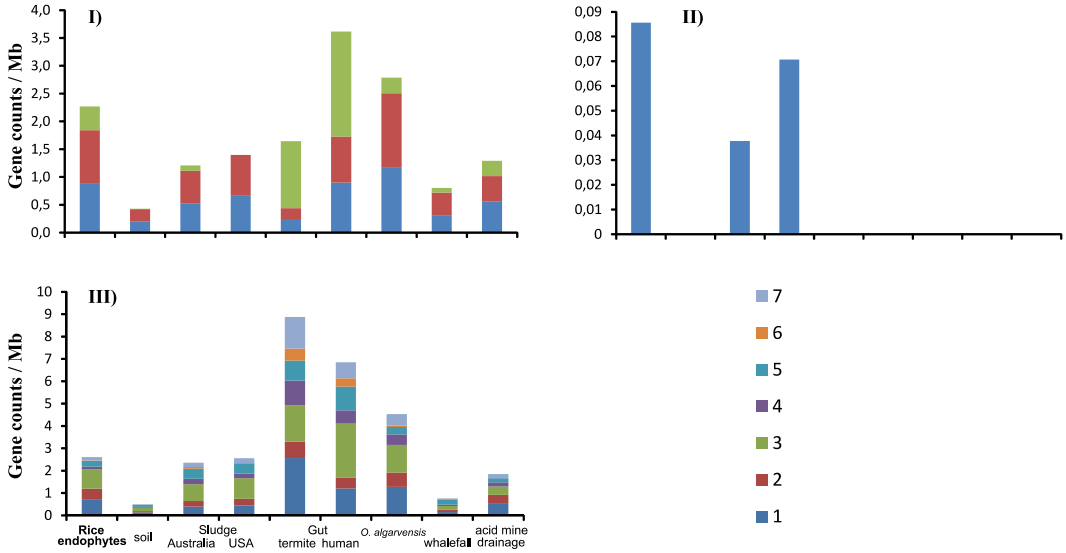

Fig. 36 Distribution of genes involved in fermentation process

Analysis of proteins for alcohol dehydrogenases, fumarate respiration and fermentation pathways. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. Proteins potentially involved (**I**) alcohol dehydrogenases (**II**) fumarate respiration and (**III**) fermentation pathways (butyrate and butane-diol). Designation of domains in (**I**) 1 pfam08240, Zn-dependent alcohol dehydrogenases, class III; 2 pfam00107, Zn-dependent alcohol dehydrogenases; 3 pfam00465, Fe-dependent alcohol dehydrogenase; in (**II**) 1 pfam02300, fumarate reductase subunit C. (**III**) Comparison of pfam domains potentially involved in butyrate and butane-diol fermentations found in selected sequenced metagenomes. 1 pfam02775, Thiamine pyrophosphate enzyme, C-terminal TPP binding domain; 2 pfam02776, Thiamine pyrophosphate enzyme, N-terminal TPP binding domain; 3 pfam01842, acetolactate synthase, small subunit (EC 2.2.1.6); 4 pfam01855 pyruvate flavodoxin/ferredoxin oxidoreductase, thiamine diP-binding domain; 5. pfam00289, pyruvate carboxylase; 6 pfam02436, pyruvate carboxylase; 7 pfam01558, pyruvate ferredoxin/flavodoxin oxidoreductase.

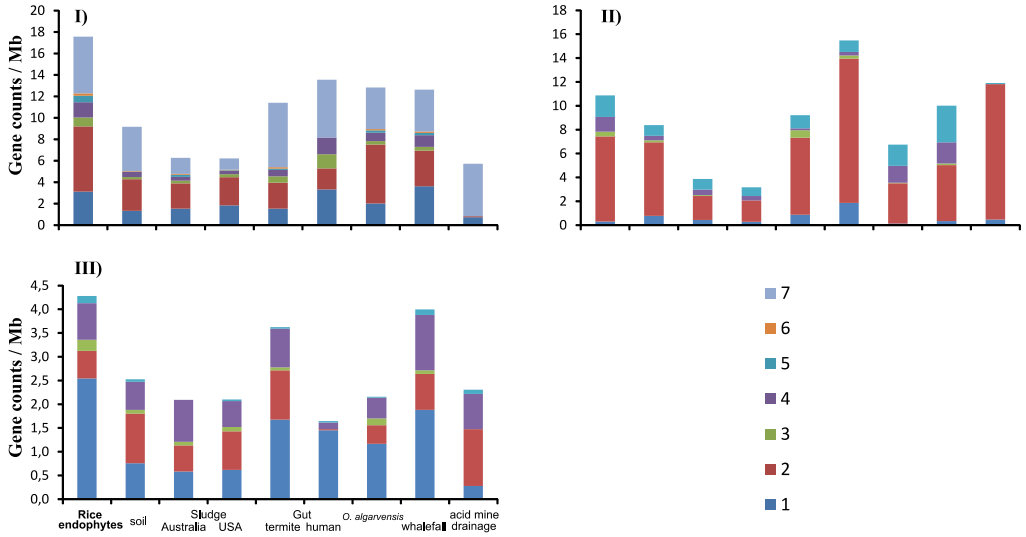

Fig. 37 Distributuion of genes involved in membrane transport

Comparison of COG categories involved in the transport of (**I**) amino acids and peptides, (**II**) sugars and (**III**) other selected compounds. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. (**I**) 1 ABC-type amino acid transport, uncharacterized (COG0765, COG4597, COG0834, COG1292, COG1279, COG0591, COG1280); 2 ABC-type branched amino acid transport (COG0410, COG0411, COG0559, COG0683, COG1114, COG4177), 3 ABCtype polar amino acids transport (COG1126), 4 ABC-type proline/glycine betaine transport (COG1125, COG1174, COG1732, COG2113, COG4175, COG4176), 5 ABC-type arginine/histidine transport (COG4160, COG4161, COG4215, COG4598), 6 ABC-type thiamine transport (COG3840, COG4143), 7 ABC-type peptide transport (COG0444, COG0601, COG0747, COG1124, COG1173, COG4166, COG4608). (**II**) 1 ABC-type polysaccharide transport, uncharacterized (COG1134, COG1682, COG4209); 2 ABC-type or uncharacterized sugar transport (COG0295, COG1129, COG1172, COG1175, COG1653, COG1869, COG1879, COG2211, COG2271, COG2610, COG2814, COG3822, COG3829, COG3833, COG4158, COG4211), 3 ABC-type xylose transport (COG4213, COG4214), 4 TRAP-type mannitol/chloroaromatic compound transport (COG4663, COG4664, COG4665, COG4666), 5 Di- and tricarboxylate transport (COG0471, COG1593, COG1638, COG2704, COG3069, COG3090). (**III**) 1 ABC-type spermidine/putrescine transport (COG1176, COG1177, COG3842); 2 ABC-type transport systems involved in resistance to organic solvents (COG0767, COG1127, COG1463, COG2854); 3 ABC-type tungstate transport (COG2998, COG4662); 4 biopolymer transport (COG0811, COG0848); 5 cyanate permease (COG2807).

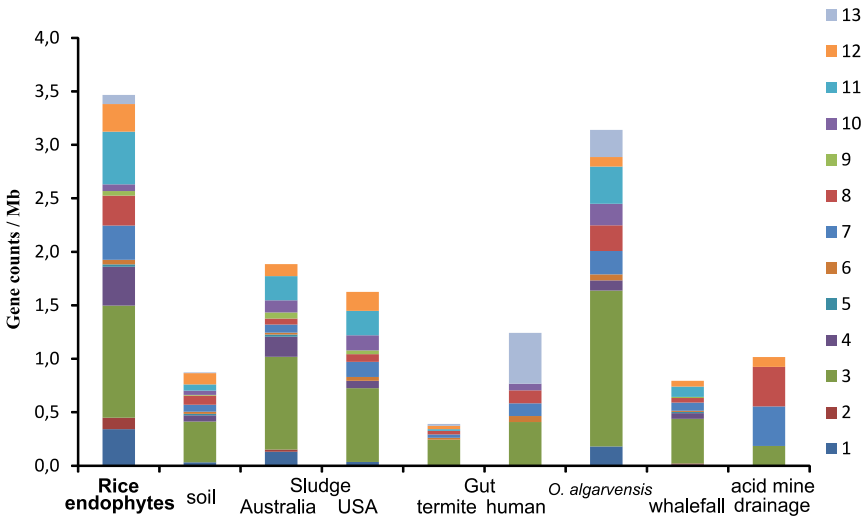

Fig. 38 Distribution of genes involved in degradation of aromatic compounds

Comparison of pfam domains potentially involved in degradation of aromatic compounds found in selected sequenced metagenomes. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. aerobic: 1 pfam00775 (dioxygenase C terminus), 2 pfam04444 (catechol dioxygenase N terminus), 3 pfam00903 (glyoxalase/bleomycin resistance protein/dioxygenase superfamily), 4 pfam02900 (catalytic LigB subunit of aromatic ring-opening dioxygenase), 5 pfam07746 (aromatic-ring-opening dioxygenase LigAB, LigA subunit), 6 pfam01361 (tautomerase enzyme), 7 pfam01188 (mandelate racemase / muconate lactonizing enzyme, C-terminal domain), 8 pfam02746 (mandelate racemase / muconate lactonizing enzyme, N-terminal domain), 9 pfam02332 (methane/phenol/toluene hydroxylase), 10 pfam04945 (YHS domain), 11 pfam00732 (GMC_oxred_N; GMC oxidoreductase), 12 pfam01738 (dienelactone hydrolase family), anaerobic: 13 pfam06050 (2-hydroxyglutaryl-CoA dehydratase, D-component).

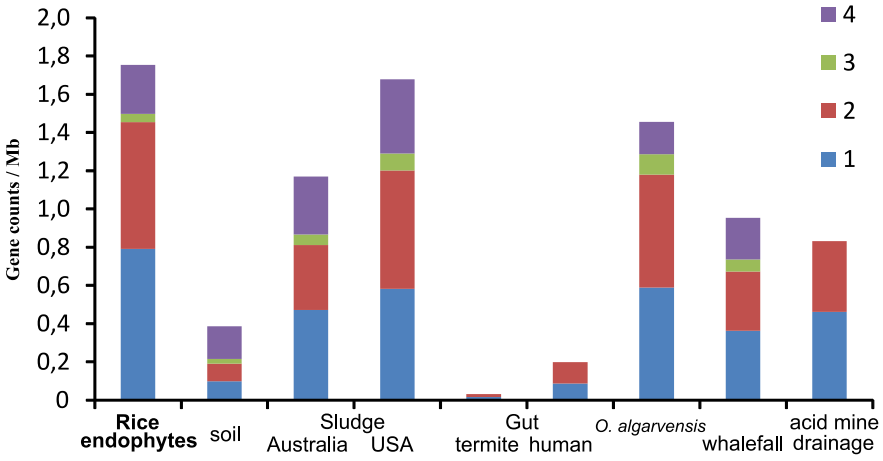

Fig. 39 Distribution of genes related to polyhydroxyalkanoates

Comparison of genes potentially involved in the synthesis and degradation of polyhydroxyalkanoates found in selected sequenced metagenomes. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. 1 pfam00108 (ketothiolase N terminus), 2 pfam02803 (ketothiolase C terminus), 3 TIGR01829 (acetoacetyl-CoA reductase), 4 COG3243 (PHA synthase).

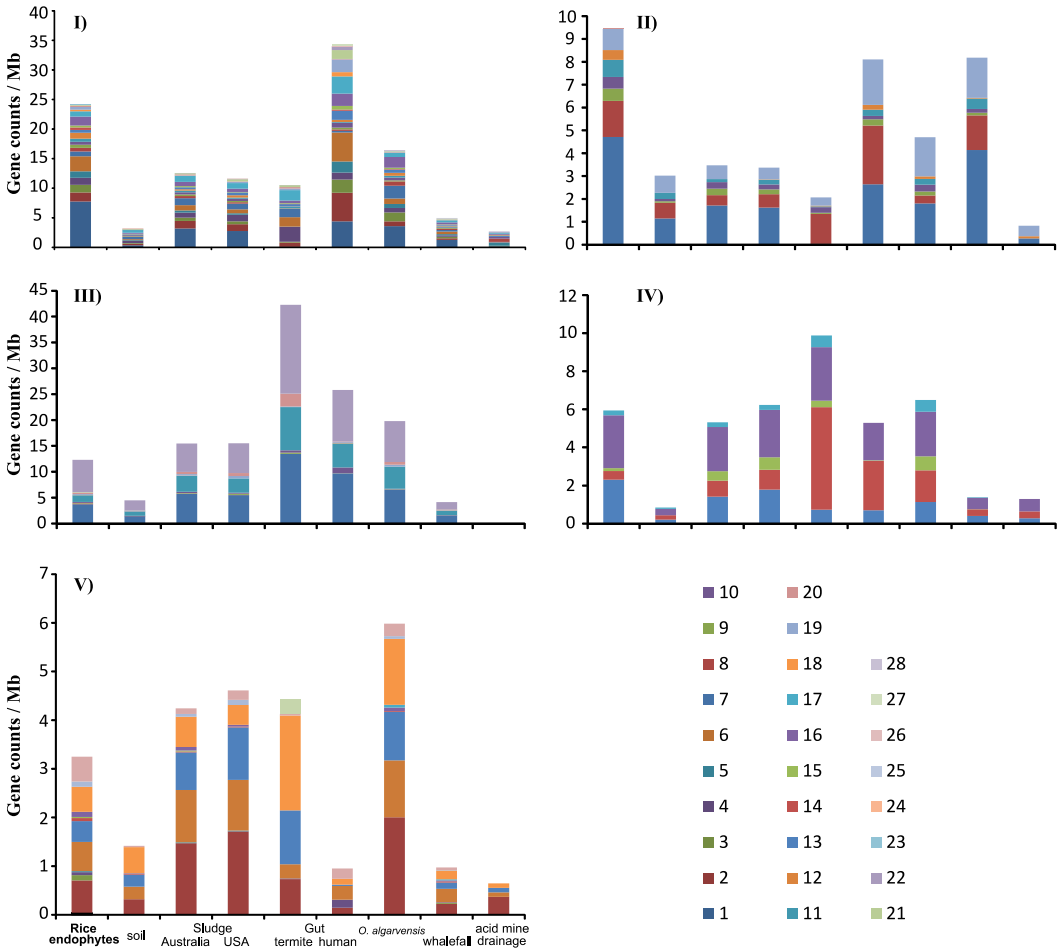

Fig. 40 Distribution of transcriptional regulator genes

Analysis of transcriptional regulators. Comparison of abundance of domains encoded in selected metagenomes, normalized as genes per Mb of metagenome sequence. For the Analysis of the transcriptional regulators different Pfam domains which define the same protein type were grouped together and named after the best characterised representative. For example: pfam00165 HTH_AraC, pfam02311 AraC_binding and pfam06719 AraC_N form the AraC-

family. For the analysis of multidomain proteins data were collected as mentioned above. To assure comparability data were not further processed but sorted according to domain composition given by the additional predicted Pfam domains, and grouped according to their in- or output domains as stated for each case. For some graphical presentations those subgroups were then further grouped into main categories, mainly according to their predicted functions. Comparison of (**I**) 1 LysR-Family: pfam00126 (HTH_1), pfam03466 (LysR_substrate), 2 pfam00486 (Trans_reg_C), 3 pfam00392 (GntR), 4 pfam00196 (YcbB), 5 pfam01047 (MarR), 6 AraC-family: pfam00165 (HTH_AraC), pfam02311 (AraC_binding), pfam06719 (AraC_N), 7 pfam00158 (Sigma54_activat), 8 pfam01037 (AsnC_trans_reg), 9 pfam00325 (Crp), 10 pfam01022 (HTH_5), 11 pfam02954 (HTH_8), 12 IclR-family: pfam01614 (IclR), pfam09339 (HTH_IclR), 13 pfam00376 (MerR), 14 pfam01638 (HxlR), 15 pfam08279 (HTH_11), 16 TetR-family: pfam00440 (TetR_N), pfam08359 (TetR_C_4), pfam08361 (TetR_C_2), pfam08362 (TetR_C_3), 17 Sigma-like: pfam00309 (Sigma54_AID), pfam00140 (Sigma70_r1_2), pfam08281 (Sigma70_r4_2), 18 pfam01418 (HTH_6), 19 DeoR-family: pfam00455 (DeoR), pfam08220 (HTH_DeoR), 20 pfam05930 (Phage_AlpA), 21 pfam04397 (LytTR), 22 pfam03551 (PadR), 23 pfam08667 (BetR), 24 pfam03704 (BTAD), 25 pfam08222 (HTH_CodY), 26 pfam08664 (YcbB), 27 pfam08769 (Spo0A_C), 28 pfam08280 (HTH_Mga); in (**II**) 1 COG0583 (LysR-family), 2 COG2207 (AraC-family), 3 pfam00325 (Crp), 4 pfam02954 (HTH_8), 5 COG1414 (IclR-family), 6 pfam01638 (HxlR), 7 COG1309 (TetR-family), 8 pfam08667 (BetR); in (**III**) 1 pfam02518 (HATPase_c), 2 pfam08521 (2CSK_N), 3 pfam02895 (H-kinase_dim), 4 pfam06580 (His_kinase), 5 pfam00512 (HisKA), 6 pfam07568 (HisKA_2), 7 pfam07730 (HisKA_3), 8 pfam01627 (Hpt) 9 pfam07536 (HWE_HK), 10 pfam00072 (Response_reg); in (**IV**) 1 pfam00563 (EAL), 2 pfam01966 (HD), 3 pfam08668 (HDOD), 4 pfam00990 (GGDEF), 5 pfam07238 (PilZ); in (**V**) 1 pfam08670 (MEKHLA), 2 pfam00989 (PAS), 3 pfam07310 (PAS_5), 4 pfam08348 (PAS_6) 5 pfam08446 (PAS_2), 6 pfam08447 (PAS_3), 7 pfam08448 (PAS_4), 8 pfam05227 (CHASE3), 9 pfam05230 (MASE2), 10 pfam05231 (MASE1), 11 pfam03707 (MHYT), 12 pfam01590 (GAF), 13 pfam04340 (DUF484), 14 pfam01614 (IclR) 15 pfam10388 (YkuI_C).

Table 8 Genes potentially involved in detoxification of ROS and products of oxygen stress

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
			Detoxification of ROS			
Catalase-related	2010560672	205	*Salmonella enterica* subsp. *enterica* serovar Dublin (ACH74415)	1E-78	148/205 (72%)	pfam06628
Catalase	2010560673	509	*Salmonella enterica* subsp. *enterica* serovar Heidelberg (ACF66546)	0.0	427/466 (91%)	pfam00199
Catalase	2010568393	249	*Paenibacillus* sp. JDR-2 (EDS55035)	5E-125	207/235 (88%)	pfam00199
Catalase	2010569673	227	*Herpetosiphon aurantiacus* ATCC 23779 (ABX02684)	2E-88	157/218 (72%)	pfam00199 pfam06628
Catalase	2010596048	48	*Bacillus* sp. SG-1 (EDL63514)	1E-04	25/53 (47%)	pfam06628
Catalase	2010596049	93	*Deinococcus geothermalis* DSM 11300 (ABF44161)	3E-26	55/73 (75%)	pfam00199
Catalase	2010604847	164	*Pseudomonas aeruginosa* PA7 (ABR85743)	4E-82	143/163 (87%)	pfam00199
Hypothetical protein	2010607047	55	*Pseudomonas fluorescens* Pf0-1 (ABA76623)	1E-10	34/39 (87%)	pfam06628
Catalase	2010609711	192	*Mycobacterium smegmatis* str. MC2 155 (ABK70727)	5E-81	138/188 (73%)	pfam00199
Catalase	2010609712	99	*Nocardia farcinica* IFM 10152 (BAD60391)	5E-33	65/81 (80%)	pfam00199
Cu/Zn superoxide dismutase	2010606458	178	*Nitrobacter hamburgensis* X14 (ABE64325)	4E-27	58/84 (69%)	pfam00080
Cu/Zn superoxide dismutase	2010608252	183	*Acinetobacter baumannii* ATCC 17978 (ABO13540)	0.073	24/45 (53%)	pfam00080
Superoxide dismutase	2010571904	160	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (ABK39737)	3E-45	93/121 (76%)	pfam00081
Superoxide dismutase	2010579060	130	*Pseudomonas putida* F1 (ABQ77146)	4E-26	71/89 (79%)	pfam00081
Superoxide dismutase	2010602615	115	*Cellvibrio japonicus* Ueda107 (ACE84205)	2E-13	35/43 (81%)	pfam00081
Superoxide dismutase	2010602616	121	*Zymomonas mobilis* subsp. *mobilis* ZM4 (AAV89684)	5E-14	34/56 (60%)	pfam00081
Superoxide dismutase	2010604119	207	*Methylococcus capsulatus* str. Bath (AAU91441)	2E-27	57/61 (93%)	pfam00081
Superoxide dismutase	2010560379	87	*Bradyrhizobium japonicum* USDA 110 (BAC53039)	3E-100	157/159 (98%)	pfam00081 pfam02777
Superoxide dismutase	2010562065	198	*Enterobacter* sp. 638 (ABP60470)	3E-41	90/122 (73%)	pfam00081 pfam02777
Superoxide dismutase	2010563525	178	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (ABR79596)	6E-84	153/160 (95%)	pfam00081 pfam02777
Superoxide dismutase	2010594372	54	*Bradyrhizobium japonicum* USDA 110 (BAC53039)	1E-118	195/198 (98%)	pfam00081 pfam02777
Superoxide dismutase	2010595481	92	*Caulobacter crescentus* NA1000 (ACL97137)	7E-88	152/164 (92%)	pfam00081 pfam02777
Superoxide dismutase	2010576155	79	*Synechococcus* sp. WH 5701 (EAQ76095)	8E-44	78/112 (69%)	pfam02777
Superoxide dismutase	2010589754	71	*Agrobacterium tumefaciens* str. C58 (AAK86683)	2E-52	97/103 (94%)	pfam02777
Superoxide dismutase	2010591394	56	*Beijerinckia indica* subsp. *indica* ATCC 9039 (ACB96131)	8E-25	76/189 (40%)	pfam02777

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
Superoxide dismutase	2010594335	74	*Methylobacterium nodulans* ORS 2060 (ACL62186)	2E-28	54/70 (77%)	pfam02777
Superoxide dismutase	2010596410	72	*Sphingomonas* sp. SKA58 (EAT09931)	9E-35	67/87 (77%)	pfam02777
Superoxide dismutase	2010596423	84	*Chlorobium tepidum* TLS (AAM72443)	2E-06	29/71 (40%)	pfam02777
Superoxide dismutase	2010601408	50	*Mesorhizobium loti* MAFF303099 (BAB54059)	3E-23	50/56 (89%)	pfam02777
Catalase (peroxidase I)	2010549739	146	*Agrobacterium tumefaciens* str. C58 (AAK88805)	6E-59	122/145 (84%)	pfam00141
Catalase (peroxidase I)	2010581995	118	*Klebsiella pneumoniae* 342 (ACI07402)	4E-34	83/100 (83%)	pfam00141
Catalase (peroxidase I)	2010597898	212	*Pseudomonas putida* W619 (ACA72735)	5E-90	190/210 (90%)	pfam00141
Catalase (peroxidase I)	2010598667	173	*Pseudomonas syringae* pv. phaseolicola 1448A (AAZ34722)	2E-56	123/171 (71%)	pfam00141
Glutathione peroxidase	2010549012	167	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (ABK38982)	1E-79	139/167 (83%)	pfam00255
Glutathione peroxidase	2010554147	155	*Aeromonas salmonicida* subsp. *salmonicida* A449 (ABO90314)	1E-38	76/94 (80%)	pfam00255
Glutathione peroxidase	2010563329	81	*Enterobacter cancerogenus* ATCC 35316 (EEA14484)	3E-31	65/77 (84%)	pfam00255
Glutathione peroxidase	2010571223	142	*Bacteroides caccae* ATCC 43185 (EDM20952)	3E-55	97/138 (70%)	pfam00255
Glutathione peroxidase	2010576010	189	*Bradyrhizobium japonicum* USDA 110 (BAC49414)	2E-77	146/189 (77%)	pfam00255
Glutathione peroxidase	2010595488	74	*Laribacter hongkongensis* HLHK9 (ACO73419)	3E-17	41/67 (61%)	pfam00255
Cytochrome c peroxidase	2010574880	167	*Pseudomonas fluorescens* Pf-5 (AAY92879)	2E-34	77/142 (54%)	pfam03150
Cytochrome c peroxidase	2010598124	280	*Mesorhizobium loti* (CAD31236)	6E-132	222/277 (80%)	pfam03150
Predicted iron-dependent peroxidase	2010560719	142	*Enterobacter cancerogenus* ATCC 35316 (EEA13397)	2E-70	127/142 (89%)	pfam04261
Predicted iron-dependent peroxidase	2010565697	97	*Enterobacter* sp. 638 (ABP61616)	5E-37	75/96 (78%)	pfam04261
Predicted iron-dependent peroxidase	2010574062	34	*Enterobacter cancerogenus* ATCC 35316 (EEA13397)	9E-12	34/34 (100%)	pfam04261
Predicted iron-dependent peroxidase	2010584370	48	*Enterobacter cancerogenus* ATCC 35316 (EEA14878)	3E-12	30/39 (76%)	pfam04261
Predicted iron-dependent peroxidase	2010593778	101	*Mycobacterium smegmatis* str. MC2 155 (ABK73115)	1E-38	77/101 (76%)	pfam04261
Predicted iron-dependent peroxidase	2010599062	169	*Klebsiella pneumoniae* 342 (ACI08688)	8E-45	94/125 (75%)	pfam04261
Tat-translocated enzyme/Dyp-type peroxidase family	2010604123	220	*Serratia proteamaculans* 568 (ABV42028)	2E-108	183/219 (83%)	pfam04261

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
Detoxification of products of oxidative stress and xenobiotics						
Glutathione S-transferase	2010549058	209	*Sinorhizobium medicae* WSM419 (ABR61946)	7E-85	150/207 (72%)	pfam02798
Glutathione S-transferase	2010549732	219	*Bradyrhizobium japonicum* USDA 110 (BAC46248)	6E-117	206/218 (94%)	pfam02798
Glutathione S-transferase	2010550013	192	*Agrobacterium tumefaciens* str. C58 (AAK86643)	6E-69	135/189 (71%)	pfam02798 pfam00043
Glutathione S-transferase	2010550238	179	*Nitrobacter winogradskyi* Nb-255 (ABA06231)	3E-74	136/178 (76%)	pfam02798
Glutathione S-transferase	2010550666	93	*Sphingomonas wittichii* RW1 glutathione S-transferase (YP_001263941)	6E-10	31/60 (51%)	pfam00043
Glutathione S-transferase	2010550981	214	*Methylocella silvestris* BL2 (ACK49724)	2E-54	115/209 (55%)	pfam02798
Glutathione S-transferase	2010552089	216	*Rhizobium leguminosarum* bv. *trifolii* WSM2304 (ACI57088)	4E-83	156/215 (72%)	pfam02798
Glutathione S-transferase	2010552309	237	*Sphingomonas* sp. RW5 (CAA12269)	6E-32	80/228 (35%)	pfam02798 pfam00043
Predicted glutathione S-transferase	2010552371	214	*Shigella dysenteriae* 1012 (EDX34138)	8E-106	178/202 (88%)	pfam00043
Glutathione S-transferase	2010553249	170	*Bradyrhizobium japonicum* USDA 110 (BAC53157)	1E-94	163/169 (96%)	pfam02798 pfam00043
Glutathione S-transferase	2010553285	211	*Bradyrhizobium japonicum* USDA 110 (BAC51065)	2E-106	185/209 (88%)	pfam00043
Glutathione S-transferase	2010553744	186	*Enterobacter* sp. 638 glutathione S-transferase (YP_001174880)	3E-15	37/48 (77%)	pfam00043
Glutathione S-transferase	2010554003	205	*Klebsiella pneumoniae* 342 (ACI08926)	7E-78	139/193 (72%)	pfam02798
Glutathione S-transferase	2010554735	64	*Bradyrhizobium japonicum* USDA 110 (BAC48204)	2E-29	62/64 (96%)	pfam02798
Glutathione S-transferase	2010556456	67	*Escherichia coli* SMS-3-5 (ACB18464)	1E-39	71/90 (78%)	pfam00043
Glutathione S-transferase	2010556457	117	*Escherichia coli* SMS-3-5 (ACB18464)	1E-39	71/90 (78%)	pfam02798
Glutathione S-transferase	2010557936	137	*Citrobacter koseri* ATCC BAA-895 (ABV15437)	4E-58	102/117 (87%)	pfam00043
Glutathione S-transferase	2010559537	102	*Bradyrhizobium japonicum* USDA 110 (BAC47773)	3E-31	63/99 (63%)	pfam00043
Glutathione S-transferase	2010559538	139	alpha proteobacterium BAL199 (EDP65418)	3E-46	77/99 (77%)	pfam02798
Glutathione S-transferase	2010561492	197	*Bradyrhizobium japonicum* USDA 110(BAC52683)	5E-100	178/182 (97%)	pfam02798 pfam00043
Glutathione S-transferase	2010563149	210	*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:-- (ABX20476)	1E-100	169/207 (81%)	pfam02798 pfam00043
Glutathione S-transferase	2010563485	165	*Enterobacter cancerogenus* ATCC 35316 (EEA12301)	8E-88	157/167 (94%)	pfam02798
Maleylacetoacetate isomerase	2010563946	94	*Polaromonas* sp. JS666 (ABE42945)	1E-23	58/84 (69%)	pfam02798
Glutathione S-transferase	2010564859	205	*Sinorhizobium medicae* WSM419 (ABR59427)	2E-55	111/184 (60%)	pfam02798 pfam00043
Glutathione S-transferase	2010566866	227	*Ralstonia eutropha* JMP134 (AAZ59526)	6E-24	70/198 (35%)	pfam02798 pfam00043
Glutathione S-transferase	2010567466	108	*Bradyrhizobium japonicum* USDA 110 (BAC52669)	2E-52	100/107 (93%)	pfam00043

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
Glutathione S-transferase	2010567808	204	*Bradyrhizobium japonicum* USDA 110 (BAC53157)	1E-106	187/203 (92%)	pfam02798 pfam00043
Maleylacetoacetate isomerase	2010567966	217	*Beijerinckia indica* subsp. *indica* ATCC 9039 (ACB94214)	8E-60	116/214 (54%)	pfam02798
Glutathione S-transferase	2010569168	242	*Chromohalobacter salexigens* DSM 3043(ABE58493)	2E-77	156/230 (67%)	pfam00043
Glutathione S-transferase	2010569299	203	*Bdellovibrio bacteriovorus* HD100 (CAE80972)	8E-41	79/191 (41%)	pfam02798 pfam00043
Glutathione S-transferase	2010569453	133	*Hoeflea phototrophica* DFL-43 (EDQ33598)	1E-46	88/130 (67%)	pfam00043
Glutathione S-transferase	2010569654	171	*Burkholderia graminis* C4D1M (EDT11420)	1E-64	120/171 (70%)	pfam02798 pfam00043
Glutathione S-transferase	2010569877	165	*Burkholderia pseudomallei* 7894 (ZP_02479799)	2E-41	80/123 (65%)	pfam02798
Glutathione S-transferase	2010571643	137	*Klebsiella pneumoniae* 342 (ACI09781)	3E-26	70/109 (64%)	pfam00043
Glutathione S-transferase	2010571688	202	*Sphingomonas* sp. RW5 (CAA12269)	3E-20	59/180 (32%)	pfam00043
Glutathione S-transferase	2010572096	101	*Rhodopseudomonas palustris* HaA2 (ABD06608)	4E-31	62/72 (86%)	pfam02798
Glutathione S-transferase	2010572155	179	*Bradyrhizobium japonicum* USDA 110 (BAC52440)	6E-89	160/167 (95%)	pfam00043
Glutathione S-transferase	2010572455	135	*Sphingopyxis alaskensis* RB2256 (ABF53851)	2E-48	90/128 (70%)	pfam02798
Glutathione S-transferase	2010573461	107	*Burkholderia graminis* C4D1M (EDT12197)	3E-36	74/102 (72%)	pfam02798
Glutathione S-transferase	2010574010	194	alpha proteobacterium BAL199 (EDP62621)	2E-35	80/177 (45%)	pfam02798 pfam00043
Glutathione S-transferase	2010576917	95	*Ochrobactrum anthropi* ATCC 49188 (ABS14707)	2E-25	56/84 (66%)	pfam00043
Glutathione S-transferase	2010577071	205	*Serratia proteamaculans* 568 (ABV40783)	6E-61	111/178 (62%)	pfam00043
Glutathione S-transferase	2010579513	110	*Azotobacter vinelandii* AvOP (EAM03791)	2E-27	62/107 (57%)	pfam00043
Glutathione S-transferase	2010579880	202	*Bradyrhizobium japonicum* USDA 110 (BAC50897)	8E-68	140/198 (70%)	pfam02798
Glutathione S-transferase	2010582672	126	*Caulobacter* sp. K31 (ABZ72563)	2E-27	71/123 (57%)	pfam00043
Glutathione S-transferase	2010583199	220	*Burkholderia xenovorans* LB400 (ABE30546)	1E-38	83/202 (41%)	pfam02798 pfam00043
Glutathione S-transferase	2010583497	155	*Agrobacterium vitis* S4 (ACM38855)	2E-67	122/148 (82%)	pfam02798
Glutathione S-transferase	2010583663	117	*Novosphingobium aromaticivorans* DSM 12444 (ABD26841)	1E-61	111/117 (94%)	pfam02798
Glutathione S-transferase	2010584670	159	*Citrobacter koseri* ATCC BAA-895 (ABV13393)	2E-52	97/144 (67%)	pfam00043
Glutathione S-transferase	2010586051	84	*Rhodospirillum rubrum* ATCC 11170 (ABC21788)	1E-25	58/82 (70%)	pfam02798
Glutathione S-transferase	2010586906	205	*Nostoc punctiforme* PCC 73102 (ACC81931)	1E-60	112/196 (57%)	pfam02798 pfam00043
Glutathione S-transferase	2010586990	102	alpha proteobacterium BAL199 (EDP65418)	8E-39	78/102 (76%)	pfam02798
Glutathione S-transferase	2010588588	201	*Klebsiella pneumoniae* 342 (ACI11760)	3E-101	182/195 (93%)	pfam02798 pfam00043
Glutathione S-transferase	2010589348	77	*Polaromonas* sp. JS666 (ABE42046)	8E-30	63/77 (81%)	pfam02798
Glutathione S-transferase	2010592424	197	*Xanthobacter autotrophicus* Py2 (ABS67969)	2E-51	107/196 (54%)	pfam02798

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
Glutathione S-transferase	2010592853	132	*Shigella flexneri* 2a str. 2457T (AAP17128)	9E-52	97/122 (79%)	pfam02798
Glutathione S-transferase	2010594021	193	*Labrenzia alexandrii* DFL-11 (EEE46383)	3E-46	96/180 (53%)	pfam02798 pfam00043
Glutathione S-transferase	2010597697	183	*Agrobacterium tumefaciens* str. C58 (AAK86935)	4E-71	127/182 (69%)	pfam02798
Maleylacetoacetate isomerase	2010597984	137	*Leptothrix cholodnii* SP-6 (ACB34357)	4E-34	68/106 (64%)	pfam02798
Maleylacetoacetate isomerase	2010598595	201	*Verminephrobacter eiseniae* EF01-2 (ABM59530)	7E-73	150/200 (75%)	pfam02798 pfam00043
Glutathione S-transferase	2010599489	100	*Erythrobacter litoralis* HTCC2594 (ABC63073)	0,0000005	25/33 (75%)	pfam02798
Glutathione S-transferase	2010600901	177	*Bordetella parapertussis* 12822 (CAE40075)	7E-57	103/162 (63%)	pfam02798 pfam00043
Glutathione S-transferase	2010601501	133	*Mesorhizobium loti* MAFF303099 (BAB54276)	2E-44	85/131 (64%)	pfam00043
Glutathione S-transferase	2010602328	117	*Pectobacterium atrosepticum* SCRI1043 (CAG73228)	6E-43	103/116 (88%)	pfam00043
Glutathione S-transferase	2010604352	183	*Erwinia tasmaniensis* Et1/99 (CAO95351)	6E-94	171/181 (94%)	pfam02798 pfam00043
Glutathione S-transferase	2010604981	119	*Bradyrhizobium japonicum* USDA 110 (BAC47768)	4E-59	112/115 (97%)	pfam02798
Glutathione S-transferase	2010605099	168	*Methylocella silvestris* BL2 (ACK50686)	2E-63	120/171 (70%)	pfam02798
Glutathione S-transferase	2010605529	189	*Polaromonas* sp. JS666 (ABE46133)	2E-44	88/165 (53%)	pfam02798
Glutathione S-transferase	2010607130	98	*Bradyrhizobium japonicum* USDA 110 (BAC53124)	5E-32	71/81 (87%)	pfam00043
Glutathione S-transferase	2010608165	149	*Pectobacterium atrosepticum* SCRI1043 (CAG73983)	7E-45	86/142 (60%)	pfam00043
Glutathione S-transferase	2010608518	143	*Rhodopseudomonas palustris* BisB5 (ABE37962)	2E-54	105/143 (73%)	pfam02798
Glutathione S-transferase	2010608649	224	*Rhizobium leguminosarum* bv. *viciae* 3841 (CAK06784)	2E-35	84/208 (40%)	pfam02798 pfam00043
Glutathione S-transferase	2010608742	167	alpha proteobacterium BAL199 (EDP63987)	3E-65	113/167 (67%)	pfam00043
Glutathione S-transferase	2010609321	218	*Sulfitobacter* sp. NAS-14.1 (EAP79659)	2E-66	104/144 (72%)	pfam02798
Glutathione S-transferase	2010609807	138	*Bradyrhizobium japonicum* USDA 110 (BAC53398)	3E-65	116/128 (90%)	pfam00043
Glutathione S-transferase	2010610795	175	*Mesorhizobium loti* MAFF303099 (BAB49280)	1E-67	118/169 (69%)	pfam00043
Glutathione S-transferase	2010611860	190	*Dechloromonas aromatica* RCB (AAZ48019)	4E-74	132/190 (69%)	pfam02798 pfam00043

Name	Gene ID	length (aa)	Closest BLASTX relative (bacterial name, acc. no.)	e-value	% identity	pfam
			Glutathione biosynthesis			
glutathione synthase	2010556337	48	*Enterobacter cancerogenus* (EEA12236)	4E-16	42/46 (91%)	pfam02955
glutathione synthase	2010556338	247	*Salmonella typhimurium* LT2 (YP_152108)	1E-124	207/224 (92%)	pfam02951 pfam02955
glutathione synthase	2010570827	250	*Pseudomonas mendocina* ymp (ABP83175)	8E-126	222/239 (92%)	pfam02951 pfam02955
glutathione synthase	2010587446	201	*Beijerinckia indica* subsp. *indica* (ACB96687)	1E-77	139/201 (69%)	pfam02951 pfam02955
glutathione synthase	2010594095	42	*Parvibaculum lavamentivorans* DS-1 (ABS65187)	6E-11	33/38 (86%)	pfam02955
glutathione synthase	2010594096	195	*Magnetospirillum magnetotacticum* (ZP_00055601)	1E-71	114/164 (69%)	pfam02951 pfam02955
glutathione synthase	2010608049	63	*Erythrobacter* sp. SD-21 (EDL50273)	2E-22	51/59 (86%)	pfam02955
glutathione synthase	2010608050	82	*Novosphingobium aromaticivorans* DSM 12444 (ABD26520)	3E-27	57/81 (70%)	pfam02955
glutamate-cysteine ligase	2010549385	244	*Enterobacter* sp. 638 (ABP61832)	1E-128	220/244 (90%)	pfam04262
glutamate-cysteine ligase	2010555118	165	*Serratia proteamaculans* 568 (ABV39953)	8E-77	145/164 (88%)	pfam04262
glutamate-cysteine ligase	2010567839	246	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (ABR78425)	1E-116	207/245 (84%)	pfam04262

Table 9 Genes potentially involved in Quorum Sensing and plant growth promotion

Name	Gene ID	Length (aa)	Closest BlastP relative (bacterial name, acc. no.)	E-value	% identit	Pfam
Autoinducer-2 (AI-2)						
S-ribosylhomocysteinase (LuxS)	2010562328	122	*Aeromonas salmonicida* subsp. salmonicida A449, ref|YP_001140608.1	$3e^{-67}$	99	PF02664
S-ribosylhomocysteinase (LuxS)	2010555119	153	*Citrobacter koseri* ATCC BAA-895, ref|YP_001455544.1	$1e^{-50}$	97	PF02664
S-ribosylhomocysteinase (LuxS)	2010555120	64	*Enterobacter* sp. 638, ref|YP_001177882.1	$7e^{-28}$	95	PF02664
S-ribosylhomocysteinase (LuxS)	2010576448	45	*Serratia marcescens*, sp|Q684Q1.1	$3e^{-13}$	97	
Periplasmic autoinducer-2-binding protein (LsrB)	2010577474	179	*Enterobacter* sp. 638, ref|YP_001178242.1	$2e^{-95}$	94	
Autoinducer-2 ABC transporter (LsrC)	2010577473	267	*Enterobacter* sp. 638, ref|YP_001178244.1	$1e^{-107}$	91	PF02653
Diffusible Signal factor (DSF)						
Enoyl-CoA hydratase, regulator of pathogenicity factor (RpfF)	2010555546	289	*Xanthomonas oryzae* pv. oryzae MAFF 311018, ref|YP_451752.1	$2e^{-167}$	99	PF00378
Sensory/regulatory protein (RpfC)	2010555548	272	*Xanthomonas oryzae,* emb|CAA66459.1	$2e^{-126}$	100	PF00072, PF02518
Sensory/regulatory protein (RpfC)	2010555547	68	*Xanthomonas oryzae* pv. oryzae PXO99A, ref|YP_001912925.1	$2e^{-30}$	100	
Two-component system response regulator (RpfG)	2010601301	158	*Xanthomonas oryzae* pv. oryzae KACC10331, ref|YP_201510.1	$3e^{-86}$	98	PF00072
Nisin and Subtilin						
Nisin leader peptide-processing serine protease (NisP)	2010602790	237	*Bacillus cereus* 03BB108, ref|ZP_03110815.1	$9e^{-122}$	99	PF00082
Nisin leader peptide-processing serine protease (NisP)	2010553616	396	*Bradyrhizobium japonicum* USDA 110, ref|NP_769684.1	0.0	93	PF00082
Lanthionine synthetase C-like protein	2010561304	310	*Kordia algicida* OT-1, ref|ZP_02160368.1	$1e^{-38}$	32	PF05147
Acylhomoserine lactone (AHL)						
N-acyl-L-homoserine lactone synthetase	2010568685	186	*Sphingomonas* sp. SKA58, ref|ZP_01303635.1	$1e^{-48}$	53	PF00765
N-acyl-L-homoserine lactone synthetase	2010589893	47	*Sinorhizobium medicae* WSM419, ref|YP_001327237.1	$8e^{-08}$	60	PF00765
N-acyl-L-homoserine lactone synthetase	2010603532	99	*Rhizobium leguminosarum* bv. trifolii WSM1325, ref|ZP_02293701.1	$5e^{-25}$	58	PF00765

Name	Gene ID	Length (aa)	Closest BlastP relative (bacterial name, acc. no.)	E-value	% identit	Pfam
N-acyl-L-homoserine lactone synthetase	2010604510	242	*Serratia marcescens*, emb|CAJ86499.1	$3e^{-127}$	96	PF00765
N-acyl-L-homoserine lactone synthetase	2010612569	143	*Sphingomonas* sp. SKA58, ref|ZP_01303635.1	$2e^{-33}$	60	PF00765
N-acyl-L-homoserine lactone synthetase	2010593852	76	*Sinorhizobium medicae* WSM419, ref|YP_001327237.1	$1e^{-27}$	77	
Autoinducer-binding transcriptional activator protein	2010549796	188	*Citrobacter koseri* ATCC BAA-895, ref|YP_001452611.1	$5e^{-79}$	72	PF03472
Autoinducer-binding transcriptional activator protein	2010553010	220	*Rhizobium leguminosarum* bv. Viciae, gb|AAO21112.1	$9e^{-21}$	31	PF00196, PF03472
Autoinducer-binding transcriptional activator protein	2010561561	260	*Pseudomonas syringae* pv. phaseolicola 1448A, ref|YP_276356.1	$5e^{-78}$	61	PF00196, PF03472
Autoinducer-binding transcriptional activator protein	2010563252	250	*Erwinia chrysanthemi*, gb|ABV57378.1	$4e^{-142}$	100	PF00196, PF03472
Autoinducer-binding transcriptional activator protein	2010564797	192	*Sinorhizobium medicae* WSM419, ref|YP_001328801.1	$2e^{-74}$	70	PF00196, PF03472
Autoinducer-binding transcriptional activator protein	2010572304	172	*Ricinus communis*, gb|EEF23550.1	$2e^{-30}$	43	PF00196, PF03472
Autoinducer-binding transcriptional activator protein	2010604830	183	*Aeromonas salmonicida* subsp. salmonicida A449, ref|YP_001143989.1	$8e^{-101}$	99	PF00196, PF03472

Genes potentially involved in PGP activities

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	pfam
			ACC deaminase			
ACC deaminase	2010580451	69	*Bradyrhizobium* sp. 278 (YP_001205796)	e^{-38}	66/69 (95%)	-
	2010580452	116		$2e^{-22}$	62/100 (62%)	-
ACC deaminase	2010583206	221	*Bradyrhizobium* sp. 278 (Y_001205796)	$2e^{-112}$	199/221 (90%)	-
ACC deaminase	2010600763	247	*Pseudomonas mendocina* ymp (YP_001188434)	$8e^{-82}$	174/240 (72%)	-
ACC deaminase	2010601152	64	*Bradyrhizobium* sp. BTAi1 (YP_001240096)	e^{-25}	48/61 (38%)	-
			IAA production			
Indole-3-pyruvate decarboxylase	2010557566	203	*Enterobacter cloacae* (AAG00523)	$4e^{-82}$	147/201 (73%)	pfam02775
Indole-3-pyruvate decarboxylase	2010608112	245	*Pantoea agglomerans (*AAB06571.1)	$4e^{-65}$	142/211 (67%)	pfam02775, pfam00205
indole-3-acetamide hydrolase	2010562414	330	*Bradyrhizobium japonicum* USDA 110 (P59385)	$3e^{-165}$	309/327 (94%)	pfam01425
indole-3-acetamide hydrolase	2010603181	240	*Brucella canis* ATCC 23365 (YP_001592436.1)	$2e^{-60}$	129/209 (61%)	pfam01425
Tryptophan monooxygenase	2010572704	243	*Pantoea agglomerans* (AAC17187)	$1e^{-09}$	49/134 (36%)	pfam01593
Tyramine oxidase	2010556753	155	*Bradyrhizobium sp.* BTAi1 (YP_001237271)	$7e^{-68}$	123/155 (79%)	pfam01179
Tyramine oxidase	2010580482	205	*Klebsiella pneumoniae subsp. pneumoniae MGH* 78578 (YP_001335129)	$1e^{-91}$	160/178 (89%)	pfam01179
			Cytokinin production			
tRNA isopentenyltransferase	2010552238	236	*Agrobacterium tumefaciens* str. C58 (NP_355007)	$4e^{-82}$	151/218 (69%)	pfam01715, pfam01745
tRNA isopentenyltransferase	2010549423	267	*Enterobacter* sp. 638 (YP_001175093)	$8e^{-140}$	236/266 (88%)	pfam01725
tRNA isopentenyltransferase	2010603162	263	*Azoarcus sp.* EbN1(YP_161051)	$4e^{-87}$	176/250 (70%)	pfam01725
			Nitrogen fixation			
NifH	2010560946	795	Bradyrhizobium sp. BTAi1, CP000494.1	0.0	703/785 (89%)	pfam00142
NifH	2010558093	657	Bradyrhizobium sp. BTAi1, CP000494.1	0.0	596/655 (90%)	pfam00142
NifH	2010586695	411	Xanthobacter autotrophicus Py2, CP000781.1	$2e^{-139}$	350/397 (88%)	pfam00142
NifH	2010589383	468	Bradyrhizobium sp. BTAi1, CP000494.1	$1e^{-172}$	419/469 (89%),	pfam00142
NifH	2010552658	198	Dickeya dadantii Ech703, CP001654.1	$3e^{-55}$	168/198 (84%),	pfam00142
NifH	2010604148	663	Clostridium botulinum A2 str. Kyoto, CP001581.1	$1e^{-68}$	423/603 (70%),	pfam00142
NifD	2010549518	492	Bradyrhizobium sp. Soph313_CPI-0246	0.0	404/411 (98%),	pfam 00148
NifD	2010549519	510	Azorhizobium caulinodans ORS 571, AP009384.1|	$7e^{-177}$	446/510 (87%)	pfam 00148
NifD	2010552331	624	Klebsiella pneumoniae, AF300326	0.0	434/455 (95%),	pfam 00148
NifD	2010586694	183	Methylocella silvestris BL2, CP001280.1	$3e^{-49}$	154/182 (84%)	pfam 00148

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	pfam
NifD	2010552333	807	Klebsiella pneumoniae, X13303.1	0.0	654/790 (82%)	pfam 00148
NifD	2010552659	510	Klebsiella pneumoniae, Y00316.1	$5e^{-159}$	433/510 (84%),	pfam 00148
NifD	2010552660	180	Dickeya dadantii Ech703	$9e^{-55}$	158/180 (87%	pfam 00148
NifD	2010598104	381	Bradyrhizobium sp. BTAi1, CP000494.1	$3e^{-130}$	319/354 (90%)	pfam 00148
NifD	2010579262	426	Rhodospirillum centenum SW, CP000613.1	$2e^{-139}$	342/384 (89%)	pfam 00148
NifK	2010598103	306	Bradyrhizobium sp. BTAi1, CP000494.1	$2e^{-100}$	268/306 (87%)	pfam 00148
NifK	2010577172	414	Desulfotomaculum acetoxidans DSM 771, CP001720.1	$1e^{-41}$	288/414 (69%)	pfam 00148
NifK	2010602178	204	Azorhizobium caulinodans ORS 571, AP009384.1	$3e^{-67}$	181/204 (88%)	pfam 00148
NifK	2010603734	708	Xanthobacter autotrophicus Py2, CP000781.1|	0.0	570/709 (80%)	pfam 00148
NifK	2010552332	855	Klebsiella pneumoniae, X13303.1	0.0	705/856 (82%),	pfam 00148
NifK	2010567499	666	Bradyrhizobium sp. BTAi1, CP000494.1	$3e^{-175}$	533/658 (81%)	pfam 00148
			Antibiotic production - Phenazine			
PhzF	2010566881	303	*Rhizobium leguminosarum* bv. viciae 3841 (YP_769590)	$4e^{-86}$	170/305 (55%)	pfam02567
PhzF	2010566965	292	*Vibrio splendidus* LGP32 (YP_002394606)	$9e^{-79}$	146/266 (54%)	pfam02567
PhzF	2010569905	198	*Bradyrhizobium japonicum* USDA 110 (NP_772283)	$7e^{-83}$	152/173 (87%)	pfam02567
PhzF	2010607877	194	*Rhodospirillum centenum* SW (YP_002298001)	$2e^{-52}$	116/196 (59%)	pfam02567
PhzF	2010613240	216	*Sagittula stellata* E-37 (ZP_01746081)	$5e^{-52}$	110/198 (55%)	pfam02567
PhzF	2010577403	222	*Pseudovibrio* sp. JE062 (YP_002683382)	$5e^{-68}$	133/222 (59%)	pfam02567
PhzF	2010592518	204	*Rhizobium leguminosarum* bv. *trifolii* WSM2304 (YP_002282736)	$5e^{-59}$	118/187 (63%)	pfam02567
PhzC	2010595601	82	*Agrobacterium vitis* S4 (YP_002550008)	$1e^{-25}$	59/82 (71%)	pfam02567
PhzC	2010597480	58	*Pseudomonas aeruginosa* 2192 (YP_002090394)	$1e^{-5}$	31/57 (54%)	-
PhzC	2010573088	121	*Rhizobium etli* CIAT 894 (ZP_03525368)	$4e^{-39}$	80/116 (68%)	pfam02567
PhzC	2010576968	101	*Dinoroseobacter shibae* DFL 12 (YP_001534079)	$3e^{-24}$	54/88 (61%)	pfam02567
			NRPS			
non-ribosomal peptide synthase	2010553934	402	*Nitrobacter* sp. Nb-311A (ZP_01046818)	$2e^{-150}$	265/376 (70%)	pfam00501
non-ribosomal peptide synthase	2010558896	331	*Escherichia fergusonii* ATCC 35469 (YP_002383617)	$6e^{-127}$	227/331 (68%)	pfam00668
non-ribosomal peptide synthase	2010561437	474	*Klebsiella pneumoniae* 342 (YP_002239777)	0.0	390/470 (82%)	pfam00501 pfam00975

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	pfam
			PKS			
PKS	2010551725	416	*Sinorhizobium medicae* WSM419 (YP_001329224)	$2e^{-112}$	204/331 (61%)	-
PKS	2010567107	120	*Paenibacillus larvae* subsp. *larvae* BRL-230010 (ZP_02327562)	$7e^{-32}$	61/114 (53%)	pfam00109
PKS	2010574831	173	*Agrobacterium vitis* S4 (YP_002551155)	$2e^{-69}$	124/159 (77%)	pfam00109
PKS	2010575684	257	*Clostridium cellulolyticum* H10 (YP_002505214)	$2e^{-67}$	135/255 (52%)	pfam02801
PKS	2010591193	229	*Chitinophaga pinensis* DSM 2588 (ZP_04359323)	$6e^{-45}$	103/225 (45%)	pfam02801
PKS	2010609173	268	*Geobacter uraniireducens* Rf4 (YP_001231833)	$2e^{-52}$	116/252 (46%)	-
PKS	2010609960	251	*Cylindrospermopsis raciborskii* AWT205 (ABX60152)	$6e^{-41}$	101/263 (38%)	pfam00698
PKS	2010612623	151	*Aspergillus fumigatus* Af293 (XP_748578)	$4e^{-12}$	48/140 (34%)	pfam00109
PKS	2010572713	238	*Clostridium beijerinckii* NCIMB 8052 (YP_001309182)	$1e^{-89}$	161/213 (75%)	pfam01323
PKS	2010591005	73	*Polaromonas* sp. JS666 (YP_549820)	$1e^{-17}$	44/60 (73%)	-
Polyketide cyclase / dehydrase	2010609973	142	*Bradyrhizobium* sp. BTAi1 (YP_001240743)	$2e^{-61}$	110/139 (79%)	pfam10604
			Siderophore - NRPS			
Phosphopantetheinyl transferase	2010549916	103	*Escherichia coli* CFT073 (NP_752599)	$7e^{-20}$	45/78 (57%)	-
Phosphopantetheinyl transferase	2010549917	122	*Citrobacter koseri* ATCC BAA-895(YP_001454125)	$7e^{-25}$	59/110 (53%)	pfam01648
Isochorismate synthase	2010592992	70	*Enterobacter cancerogenus* ATCC 35316 (ZP_03283415)	$8e^{-31}$	63/70 (90%)	pfam00425
Isochorismate synthase	2010573702	130	*Enterobacter cancerogenus* ATCC 35316 (ZP_03283415)	$3e^{-62}$	116/130 (89%)	-
Isochorismate synthase	2010596433	129	*Serratia proteamaculans* 568 (YP_001479647)	$4e^{-46}$	89/123 (72%)	pfam00425
Isochorismatase family	2010560386	232	*Bradyrhizobium japonicum* USDA 110 (NP_769365)	$1e^{-125}$	219/232 (94%)	pfam00857
Isochorismatase family	2010564462	304	*Bradyrhizobium* sp. BTAi1 (YP_001240733)	$1e^{-115}$	204/299 (68%)	pfam00857 pfam08450
Isochorismatase family	2010565050	232	*Ralstonia eutropha* H16 (YP_841456)	$2e^{-115}$	193/228 (84%)	pfam00857
Isochorismatase family	2010568191	180	*Enterobacter* sp. 638 (YP_001174899)	$2e^{-87}$	151/179 (84%)	pfam00857
Isochorismatase family	2010571597	86	*Burkholderia thailandensis* MSMB43 (ZP_02466516)	$1e^{-15}$	42/84 (50%)	pfam00857
Isochorismatase family	2010573387	131	*Pseudomonas syringae* pv. Tomato T1 (ZP_03398237)	$3e^{-43}$	83/131 (63%)	pfam00857
Isochorismatase family	2010575820	218	*Agrobacterium radiobacter* K84 (YP_002542428)	$1e^{-96}$	171/214 (79%)	pfam00857
Isochorismatase family	2010582078	48	*Leptospirillum* sp. Group II '5-way CG' (EDZ40429)	$1e^{-4}$	24/48 (50%)	pfam00857
Isochorismatase family	2010582147	91	*Verrucomicrobium spinosum* DSM 4136 (ZP_02927524)	$3e^{-37}$	72/87 (82%)	pfam00857
Isochorismatase family	2010584189	166	*Bacillus cereus* 95/8201 (ZP_04249212)	$8e^{-85}$	152/155 (98%)	pfam00857
Isochorismatase family	2010587467	68	*Sagittula stellata* E-37 (ZP_01744167)	$1e^{-23}$	54/66 (81%)	pfam00857
Isochorismatase family	2010589847	104	*Burkholderia glumae* BGR1 (YP_002907992)	$1e^{-22}$	57/98 (58%)	pfam00857
Isochorismatase family	2010591328	189	*Citrobacter* sp. 30_2 (YP_002849831)	$7e^{-90}$	158/188 (84%)	pfam00857
Isochorismatase family	2010591571	84	*Rhodospirillum rubrum* ATCC 11170 (YP_428084)	$3e^{-21}$	47/78 (60%)	pfam00857

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	pfam
Isochorismatase family	2010594535	167	*Ochrobactrum anthropi* ATCC 49188 (YP_001371161)	$2e^{-70}$	124/159 (77%)	pfam00857
Isochorismatase family	2010595378	161	*Agrobacterium tumefaciens* str. C58 (NP_355032)	$8e^{-57}$	98/144 (68%)	pfam00857
Isochorismatase family	2010596424	85	*Synechococcus* sp. JA-2-3B'a(2-13) (YP_477042)	$7e^{-23}$	54/74 (72%)	pfam00857
Isochorismatase family	2010598762	130	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 (YP_857194)	$8e^{-16}$	44/55 (80%)	pfam00857
Isochorismatase family	2010601063	98	*Rhizobium leguminosarum* bv. trifolii WSM1325 (ZP_02295199)	$3e^{-36}$	70/98 (71%)	pfam00857
Isochorismatase family	2010611393	182	*Solibacter usitatus* Ellin6076 (YP_822194)	$3e^{-58}$	111/174 (63%)	pfam00857
Isochorismatase family	2010611617	80	*Bradyrhizobium japonicum* USDA 110 (NP_771189)	$8e^{-23}$	52/55 (94%)	pfam00857
Isochorismatase family	2010612545	172	*Bacillus thuringiensis* serovar pondicheriensis BGSC 4BA1 (ZP_04090284)	$6e^{-49}$	93/170 (54%)	pfam00857
			Siderophore - NIS			
alcaligin biosynthesis protein	2010552906	306	*Nitrobacter* sp. Nb-311A (ZP_01047028)	$2e^{-131}$	225/305 (73%)	pfam10331
alcaligin biosynthesis protein	2010586965	227	*Variovorax paradoxus* S110 (ZP_03550986)	$1e^{-74}$	132/168 (78%)	pfam10331
Siderophore synthetase component	2010605844	228	*Escherichia fergusonii* (AAL01537)	$1e^{-114}$	200/214 (93%)	pfam04183
Siderophore synthetase component	2010607044	270	*Staphylococcus aureus* RF122 (YP_415575)	$1e^{-18}$	74/247 (29%)	pfam04183
Siderophore synthetase component	2010609667	177	*Erwinia tasmaniensis* Et1/99 (YP_001908945)	$6e^{-90}$	157/174 (90%)	pfam04183
Siderophore synthetase component	2010611486	206	*Methylobacterium nodulans* ORS 2060	$8e^{-41}$	99/199 (49%)	pfam04183

* NifH= Nitrogenase iron protein subunit, NifH (EC 1.18.6.1); NifD= Nitrogenase molybdenum-iron protein, NifD; NifK= Nitrogenase molybdenum-iron protein, NifK; PhzF= phenazine biosynthesis protein PhzF family; PhzC= Predicted epimerase, PhzC/PhzF homolog; PKS= Polyketide synthase modules and related proteins; Phosphopantetheinyl= Phosphopantetheinyl transferase component of siderophore synthetase

Table 10 Genes potentially involved in anaerobic metabolism

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	Pfam/COG
			Alcohol dehydrogenases			
Zn-dependent	2010551821	339	*Enterobacter* sp. 638, YP_001178360.1	$8e^{-169}$	89	pfam08240, pfam00107
Zn-dependent (class III)	2010553457	168	*Klebsiella pneumoniae* subsp. MGH 78578, YP_001335613.1	$5e^{-81}$	98	pfam08240
Zn-dependent	2010553810	50	*Enterobacter sakazakii* ATCC BAA-894, YP_001437772.1	$2e^{-06}$	92	pfam08240
Zn-dependent	2010553811	265	*Enterobacter sakazakii* ATCC BAA-894, YP_001437772.1	$5e^{-134}$	87	pfam00107
Zn-dependent	2010554064	159	*Bradyrhizobium japonicum* USDA 110, NP_772855.1	$9e^{-48}$	82	COG1064
Zn-dependent (class III)	2010554304	42	*Bradyrhizobium japonicum* USDA 110, NP_772855.1	$4e^{-18}$	97	COG1062
Fe-dependent	2010556071	126	*Salmonella enterica* subsp. enterica serovar Hadar str. RI_05P066, ZP_02684855.1	$1e^{-55}$	87	COG1979
Fe-dependent	2010556072	198	*Enterobacter* sp. 638, YP_001178132.1	$3e^{-105}$	94	COG1979
Zn-dependent	2010557234	79	*Sinorhizobium medicae* WSM419	$2e^{-15}$	67	COG 1064
Zn-dependent	2010557236	151	*Bradyrhizobium japonicum* USDA 110	$1e^{-15}$	71	pfam00107
Zn-dependent	2010557515	134	*Yersinia enterocolitica* subsp. *enterocolitica* 8081 YP_001007877.1	$9e^{-58}$	77	pfam08240
Zn-dependent	2010558753	77	*Stappia aggregata* IAM 12614, ZP_01549907.1	$4e^{-25}$	68	pfam08240
Zn-dependent (class III)	2010559156	109	*Sinorhizobium meliloti* 1021, NP_436398.1	$8e^{-45}$	82	COG1062
Zn-dependent (class III)	2010559157	100	*Sinorhizobium meliloti* 1021, NP_436710.1	$3e^{-51}$	96	COG1062
Zn-dependent	2010559588	37	*Chthoniobacter flavus* Ellin428, ZP_03133032.1	$5e^{-04}$	58	COG 1064
Zn-dependent	2010560914	121	ZP_00628790.1	$1e^{-43}$	71	COG 1064
Zn-dependent	2010564667	130	*Bradyrhizobium japonicum* USDA 110, NP_771918.1	$3e^{-45}$	95	pfam08240
Zn-dependent	2010565265	171	*Bradyrhizobium japonicum* USDA 110, NP_772295.1	$2e^{-91}$	96	COG 1064
Zn-dependent	2010565266	50	*Bradyrhizobium japonicum* USDA 110, NP_772295.1	$1e^{-05}$	83	pfam00107
Zn-dependent	2010569502	194	*Bradyrhizobium japonicum* USDA 110, NP_772710.1	$6e^{-94}$	90	pfam00107
Zn-dependent	2010571036	81	*Ochrobactrum anthropi* ATCC 49188, YP_001372437.1	$1e^{-17}$	71	pfam08240
Zn-dependent	2010575520	92	*Rhodopseudomonas palustris* HaA2, YP_486964.1	$8e^{-40}$	79	pfam08240
Zn-dependent	2010575991	170	*Rhizobium leguminosarum* bv. trifolii WSM2304, YP_002280351.1	$3e^{-96}$	99	pfam00107
Zn-dependent	2010576144	216	*Bradyrhizobium japonicum* USDA 110, NP_774538.1	$6e^{-108}$	99	COG1062
Zn-dependent	2010576966	181	*Mesorhizobium* sp. BNC1, YP_674817.1	$6e^{-66}$	75	pfam08240
Zn-dependent	2010577953	104	*Bradyrhizobium* sp. ORS278, YP_001207379.1	$3e^{-45}$	100	pfam08240
Zn-dependent	2010578537	240	*Brucella melitensis* 16M, NP_540736.1	$2e^{-117}$	88	pfam00107

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	Pfam/COG
Zn-dependent	2010578704	243	*Methylocella silvestris* BL2, YP_002360545.1	$5e^{-77}$	76	pfam08240
Zn-dependent	2010579529	102	*Enterobacter* sp. 638, YP_001178360.1	$3e^{-43}$	96	pfam08240
Fe-dependent	2010580442	152	*Serratia proteamaculans* 568, YP_001480437.1	$3e^{-54}$	66	pfam00465
Zn-dependent (class III)	2010581499	70	*Brevundimonas* sp. BAL3, EDX80816.1	$3e^{-21}$	78	pfam08240
Zn-dependent	2010581823	134	*Bradyrhizobium japonicum* USDA 110, NP_769420.1	$6e^{-61}$	80	pfam08240
Fe-dependent	2010588471	213	*Aeromonas hydrophila*, AAK71638.1	$7e^{-74}$	66	pfam00465
Zn-dependent	2010592153	248	*Bradyrhizobium japonicum* USDA 110, NP_770741.1	$3e^{-128}$	96	pfam08240, pfam00107
Zn-dependent	2010600779	124	*Agrobacterium tumefaciens* str. C58, NP_353648.1	$8e^{-46}$	80	pfam00107
Fe-dependent	2010602284	206	*Clostridium thermocellum* ATCC 27405, YP_001036535.1	$2e^{-68}$	66	Pfam00465
Zn-dependent	2010603042	36	*Sinorhizobium meliloti* 1021, NP_386957.1	$5e^{-09}$	80	Pfam00107
Zn-dependent	201060332	46	*Rhodopseudomonas palustris* CGA009, NP_946027.1	$4e^{-10}$	69	Pfam08240
Zn-dependent	2010608824	139	*Paracoccus denitrificans* PD1222, ZP_00628790.1	$2e^{-39}$	70	Pfam08240
Zn-dependent	2010608829	119	*Novosphingobium aromaticivorans* DSM 12444, YP_497148.1	$2e^{-42}$	72	Pfam08240
Zn-dependent	2010610783	96	*Oceanobacillus iheyensis* HTE831, NP_693737.1	$2e^{-28}$	58	Pfam08240
Zn-dependent (class III)	2010611677	82	Sinorhizobium meliloti 1021, NP_385076.1	$7e^{-35}$	89	COG1062
			Butane-diol fermentation			
ALS	2010551065	156	*Brucella melitensis* 16M, NP_541329.1	$2e^{-50}$	86	pfam02776
ALS	2010551409	89	*Enterobacter* sp. 638, YP_001178720.1	$6e^{-35}$	84	COG3978
ALS	2010555337	75	*Aeromonas salmonicida* subsp. salmonicida A449, YP_001143126.1	$4e^{-34}$	100	pfam01842
ALS	2010555773	94	*Salmonella enterica* subsp. enterica serovar Typhi str. CT18, NP_458142.1	$4e^{-38}$	79	pfam01842
ALS	2010560364	101	*Enterobacter cancerogenus* ATCC 35316, ZP_03283882.1	$8e^{-45}$	97	pfam01842
ALS	2010560365	55	*Escherichia coli* O157:H7, ZP_03086248.1	$4e^{-20}$	92	pfam02776
ALS	2010560366	99	*Enterobacter* sp. 638, YP_001174766.1	$4e^{-48}$	94	pfam02776
ALS	2010561390	163	*Escherichia coli* APEC01, YP_851279.1	$2e^{-84}$	95	pfam01842
ALS	2010561391	130	*Salmonella enterica* subsp. enterica serovar Typhi str. J185, ZP_03377681.1	$5e^{-61}$	93	pfam02775
ALS	2010561392	444	*Enterobacter cancerogenus* ATCC 35316, ZP_03280796.1	0	91	pfam02775, pfam02776
ALS	2010563127	148	*Bradyrhizobium japonicum* USDA 110, NP_773143.1	$1e^{-79}$	95	pfam02775
ALS	2010563128	180	*Bradyrhizobium japonicum* USDA 110, NP_773141.1	$7e^{-94}$	95	pfam01842
ALS	2010569834	236	*Methylobacterium extorquens* PA, YP_001642039.1	$6e^{-76}$	65	pfam02776

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	Pfam/COG
ALS	2010570097	82	*Ochrobactrum anthropi* ATCC 49188, YP_001372613.1	$9e^{-32}$	84	pfam02776
ALS	2010570304	137	*Beijerinckia indica* subsp. indica ATCC 9039, YP_001833518.1	$3e^{-51}$	72	pfam02775
ALS	2010570306	45	*Rhizobium leguminosarum* bv. trifolii WSM1325, ZP_02295678.1	$2e^{-12}$	78	pfam02776
ALS	2010573069	243	*Stappia aggregata* IAM 12614, ZP_01550998.1	$4e^{-85}$	62	pfam02776
ALS	2010574820	93	J*anthinobacterium* sp. Marseille, YP_001353840.1	$1e^{-43}$	91	pfam02775
ALS	2010574821	123	*Cupriavidus taiwanensis,* YP_002005052.1	$4e^{-58}$	95	pfam01842
ALS	2010575422	54	Ochrobactrum anthropi ATCC 49188, YP_001369956.1	$7e^{-12}$	58	pfam02776
ALS	2010575724	93	*Sinorhizobium meliloti* 1021, NP_384673.1	$2e^{-36}$	80	pfam02776
ALS	2010578710	105	*Sinorhizobium meliloti* 1021, NP_386220.1	$6e^{-54}$	93	pfam02776
ALS	2010580908	96	*Escherichia coli* O157:H7 EDL933, NP_290309.1	$4e^{-50}$	100	pfam01842
ALS	2010580909	151	*Shigella dysenteriae* 1012, ZP_03063332.1	$3e^{-70}$	100	pfam02776
ALS	2010583320	80	*Sphingopyxis alaskensis* RB2256, YP_616517.1	$6e^{-27}$	72	pfam01842
ALS	2010584592	85	*Dechloromonas aromatica* RCB, YP_284837.1	$1e^{-32}$	77	pfam01842
ALS	2010584593	144	*Azoarcus* sp. EbN1, YP_159712.1	$1e^{-52}$	77	pfam02776
ALS	2010590334	104	*Parvibaculum lavamentivorans* DS-1, YP_001413730.1	$2e^{-46}$	84	pfam02776
ALS	2010590335	91	*Rhodospirillum rubrum* ATCC 11170, YP_425560.1	$4e^{-36}$	83	pfam01842
ALS	2010590336	41	*Rhodospirillum rubrum* ATCC 11170, ZP_02187332.1	$6e^{-10}$	68	pfam02776
ALS	2010601054	47	*Bradyrhizobium japonicum* USDA 110, NP_769605.1	$1e^{-18}$	97	pfam02776
ALS	2010601066	218	*Citrobacter koseri* ATCC BAA-895, YP_001451717.1	$1e^{-91}$	73	pfam02776
ALS	2010602502	163	*Beijerinckia indica* subsp.indica ATCC 9039, YP_001833517.1	$9e^{-69}$	79	pfam01842
			Butyrate fermentation			
PDC	2010557566	203	*Citrobacter koseri ATCC BAA-895*, YP_001452002.1	$3e^{-80}$	73	pfam02775
PFOR	2010560642	125	*Escherichia coli* UMN026, YP_002412386.1	$3e^{-63}$	95	pfam02775
PFOR	2010562116	410	*Klebsiella pneumoniae,* CAA31501.1	$8e^{-180}$	76	pfam01558
PFOR	2010569109	80	*Aeromonas hydrophila subsp. hydrophila ATCC 7966,* YP_856035.1	$1e^{-11}$	86	COG1013
PFOR	2010569762	234	*Magnetococcus sp. MC-1,* YP_865656.1	$6e^{-88}$	64	COG1013
PFOR	2010569763	212	*Magnetococcus sp. MC-1,* YP_865655.1	$5e^{-78}$	68	pfam01558
PFOR	2010572482	197	*Bradyrhizobium sp. ORS278,* YP_001207685.1	$7e^{-68}$	73	pfam02775
PFOR	2010575893	159	*Enterobacter sakazakii ATCC BAA-894,* YP_001437774.1	$8e^{-85}$	93	pfam01558
PFOR	2010584796	206	*Paenibacillus larvae subsp. larvae BRL-230010,* ZP_02330685.1	$2e^{-19}$	37	pfam01855
PFOR	2010587454	241	*Clostridium botulinum F str. Langeland,* YP_001392038.1	$3e^{-99}$	76	pfam01558
PFOR	2010589689	77	*Acidobacteria bacterium Ellin345,* YP_589809.1	$2e^{-29}$	77	pfam01855

Name*	Gene ID	Length (aa)	Closest BlastP relative	e-value	% identity	Pfam/COG
PFOR	2010591972	102	*Klebsiella pneumoniae,* CAA34396.1	$1e^{-37}$	91	pfam01855
PFOR	2010592689	108	*Verrucomicrobiae bacterium DG1235,* EDY80517.1	$1e^{-35}$	76	pfam01855
PFOR	2010593498	139	*Bradyrhizobium japonicum USDA 110,* NP_769498.1	$3e^{-55}$	94	pfam02775
PFOR	2010594200	222	*Pseudomonas mendocina ymp,* YP_001188363.1	$3e^{-66}$	59	pfam01558
PFOR	2010595323	172	*Klebsiella pneumoniae 342,* YP_002237566.1	$5.e^{-74}$	82	COG1013
PFOR	2010604094	184	*Bradyrhizobium japonicum USDA 110,* NP_773383.1	$3e^{-101}$	97	COG1014
PFOR	2010607410	225	*Erythrobacter litoralis HTCC2594*	$8e^{-79}$	74	COG1013
Pyruvate carboxylase	2010564796	202	*Rhizobium sp. TAL1145*	$4e^{-98}$	92	pfam 00289, pfam 02786
Pyruvate carboxylase	2010580063	167	*Rhizobium leguminosarum bv. trifolii WSM2304*	$4e^{-72}$	88	pfam02436
			Fumarate respiration			
FRD	2010552412	105	*Escherichia albertii* TW07627, ZP_02901296.1	$2e^{-142}$	78	pfam02300
FRD	2010569258	105	*Escherichia albertii* TW07627, ZP_02901296.1	$2e^{-162}$	80	pfam02300
FRD	2010583689	132	*Aeromonas hydrophila* subsp. *hydrophila*, YP_857701.1	$8e^{-70}$	96	pfam02300
FRD	2010600597	125	*Chromobacterium violaceum* ATCC 12472, NP 903037	$2e^{-20}$	51	pfam02300

*ALS= Acetolactate synthase small/large subunit, EC 2.2.1.6; PDC= Pyruvate decarboxylase; PFOR= Pyruvate:ferredoxin oxidoreductase and related 2-oxoacid:ferredoxin oxidoreductases; FRD= Fumarate reductase subunit C

Chapter 8

Summary and concluding remarks

Terrestrial plants have a long history of interactions with microorganisms. In fact, it has been hypothesized that primitive plants only colonized dry land after establishing symbiosis with arbuscular mycorrhizal fungi, which helped them with the acquisition of water and nutrients from soil (Harrison, 2005). Plant-bacterium symbioses may date even from earlier ages and are at the very root of our existence (Cavalier-Smith, 2010). Today, we are aware that plant-microbe symbioses are innate to each host plant species on Earth and interactions between plants and microorganisms are the rule rather than the exception. With their capacity to synthesize a vast range of photoassimilates, plants sustain a diverse number of heterotrophic organisms, including bacteria, which are attracted by the release of a rich source of carbonaceous compounds and may directly interact with the host plants. Specialized bacteria might even colonize the internal plant tissues, thus becoming endophytic ('endo', inside; 'phyte', plant). Despite the advances from studies assessing the bacterial diversity and community compostion of endophytes, little is known about the ecology of these. In other words: how do they interact with their host plants? What are the factors affecting these interactions? What are they doing inside the plant?

We do known that a vast number of bacteria found to be associated with host plants, including the ones isolated in this study, have more than one type of PGP properties. Thus, strains selected on the basis of their presumed beneficial activities with the host plant are often used for inoculation assays, in which many introduced bacteria have been shown to be capable of promoting plant growth under greenhouse conditions. However, they often fail to deliver similar results when applied in open fields, thus hindering our ability to promote sustainable agriculture on the basis of inoculation with natural agents. Lack of competitive abilities against indigenous rhizobacteria and/or endophytic communities is often the presumed cause of failure (Compant et al., 2010; Lugtenberg & Kamilova, 2009). Hence, a better understanding of the colonization process and the factors that directly affect the bacterial competition in the rhizosphere are major requirements to foster our knowledge in order to promote a more environmentally-friendly agriculture.

The research described in this thesis aimed to identify the adaptive and plant-beneficial mechanisms that characterize competent endophytes and to assess the importance of the mechanisms found for the establishment of beneficial mutualistic relationships. A

comprehensive discussion on how plants can benefit from their associated bacteria is given in **Chapter 1**, whereas in **Chapter 2** we discuss what is needed for a bacterium to become a 'competent endophyte'. For instance, chemotaxis towards plant exudates, osmotic protection and resistance to oxidative stresses, dedicated communication systems via autoinducer molecules and transcriptional regulators, host recognition and adhesion outer-membrane proteins and production of hydrolytic enzymes are all colonization and plant-adaptive traits that will certainly favour particular soil-dwelling bacteria to outcompete others. However, the second partner may also have a say, and it is most likely that plants will select for (or benefit from) bacteria that harbour advantageous PGP properties, thus establishing synergistic interactions. Probably, one of the best PGP functions of endohpytes, which is often overlooked and taken for granted, is the removal of metabolic waste products. Hence, competent endophytes may be defined as those bacteria that reveal some of the aforementioned key properties, allowing them to increase plant fitness. The resulting interaction is a win-win relationship, in which host plants benefit by increasing their growth and the endophytes benefit from the reliable nutrient sources offered by the plant. Many of these interactions directly affect plant physiology via the production and modulation of phytohormones. There are, at least, three mechanisms that exemplify how plant-endophytes interact synergistically:

- Holland (1997) proposed that the phytohormones CKs are produced by microbial symbionts to stimulate plant growth. As a consequence of growth, some waste metabolites are generated and used by the bacteria for their own growth. Production of IAA and CKs by endophytic bacteria often induces plant growth. To form new tissue, plant cell walls are degraded, releasing methanol and other waste products. Therefore, methylotrophic bacteria and many others waste scavengers might induce plant growth to harvest those metabolites, thus improving their own growth.
- Glick & colleagues (1998) described a model in which rhizosphere bacteria containing ACC deaminase can improve plant growth by modulating plant ET levels, thus ameliorating environmentally-induced stresses. In **Chapter 2**, I expanded this function to endophytic bacteria. Under stress conditions, host plant genes involved in the synthesis of ET are induced and the immediate precursor of ET, ACC, is secreted from the host cells to be converted into ET. In the presence of ACC deaminase-containing bacteria, this ACC is taken up and used as a carbon source (ACC → NH_3 + α-ketobutyrate), thereby reducing the amount of ET produced. As probably not all ACC is taken up by the bacteria, plants are still capable of modulating their own growth and

development without the risk of overproducing ET and the bacteria benefit from the available nutrient source.

- Taghavi & colleagues (2010) recently showed how *Enterobacter* sp. 638 stimulates the growth of poplar trees. By sequencing the whole genome of *Enterobacter* sp. 638, the authors found a genomic region with genes involved in plant growth promotion and sucrose metabolism. Transcriptome and metabolic analyses revealed that the synthesis of VOCs acetoin and 2,3-butanediol was induced by the uptake of sucrose. These VOCs are phytohormones involved in plant growth promotion and are also related to ISR. Hence, uptake of sucrose favours bacterial growth and increases the production of VOCs, which induces host growth.

Plants, as they grow, synthesise a vast number of photoassimilated compounds. It is assumed that most of the metabolic products are exuded via the root tissue, which process directly influences the biological processes that occur in the plant surroundings. Plants are constantly exposed to often fluctuating environmental factors, which directly affect their physiology and metabolic processes, and therefore any factors that bear on this will affect the plant physiological status. Thus, soil type, agriculture management regime, plant growth stage, abiotic and biotic stresses and many other factors will have a direct influence on plant physiology. In the light of the tight connection between plant physiology and the make-up of the associated bacterial communities, I posit that the effects of these factors count for the plant-associated and endophytic bacterial community alike. Given the foregoing, we investigated the effect of plant genotype, soil type and nutrient use efficiency on the composition of different bacterial communities associated with the root tissues of ten rice cultivars (**Chapter 3**). A significant correlation with the composition of total bacteria, *Alpha*- and *Beta-proteobacteria*, *Pseudomonas* was observed for rice genotype (cultivar type). Only in one case, the community of *Actinobacteria,* there was a better correlation with soil type. It is interesting to note that cultivars belonging to *Oryza sativa* subspecies *indica* tended to select similar bacterial communities, whereas those belonging to subspecies *japonica* and *aromatica* selected ones with divergent community structures. This is most likely explained by a difference in physiological characteristics dictated by the host genetic make-up. For instance, *indica* cultivars have been bred to achieve high crop yields, whereas *japonica* cultivars have been kept with higher genetic variation for use for production under adverse environmental conditions. Therefore, one might speculate that both strategies yield distinct bacterial communities.

One of the previously investigated rice cultivars, denoted APO, was further used to assess the rice root endophytic community by culture-dependent and -independent approaches (**Chapter 4**). Phylogenetic analysis using a 16S rRNA gene clone library revealed a diversity of prokaryotes encompassing 16 phyla/classes, while the community assessed by isolation was limited to 5 classes. However, it was congruent with the clone sequences and represented around 33% of that assessed by the clone library. To the best of our knowledge, this study is the most comprehensive survey of uncultured and cultured endophytes so far, which greatly expands the range of known rice endophytes. *Gammaproteobacteria* was by far the largest class, with members of *Enterobacter* being the most abundant in both approaches. These results were confirmed by metagenome analysis (**Chapter 7**) and suggest a high specificity of *Enterobacter* spp. to rice plants of cultivar APO. Analyses of bacterial plant-adaptive and PGP properties showed that many of the selected strains possess more than one activity, which hints at the assumption that endophytes are highly adapted and thrive inside the host plant, improving their growth. In addition, the results from C utilization profiles indicate that various trophic levels in a food network co-evolve, which might also explain the astonishing diversity found inside the rice roots.

Further characterization of the *Enterobacter*-related strains resulted in the identification of two novel species, with the proposed names *Enterobacter oryziphilus* (strain REICA_142^T) and *Enterobacter oryzendophyticus* (strain REICA_082^T) (**Chapter 5**). Both strains revealed a plethora of PGP properties and they were rather closely related, on the basis of their 16S rRNA and *rpoB* genes, to other PGP *Enterobacter* species. Introduction of *E. oryziphilus* strain REICA_142^T onto rice revealed an (insignificant) increase of 35% in plant FW when rice plants were cultivated in non-sterile soil. These results suggest that *E. oryziphilus* is adapted to the rice plant environment and might promote plant growth under certain conditions, although the exactly mechanism is hitherto unclear. As exemplified above, *Enterobacter* might increase plant growth by various mechanisms, but our strains show high activity of fixation of N_2 *in vitro*. This is an energetically highly demanding process, which only occurs when certain conditions are met. Under flood conditions and in the presence of reliable nutrient sources, *Enterobacter* might actively incorporate atmospheric N_2 into the rice host metabolism, like rhizobia do inside leguminous plants. Although speculative, this assumption is worth of further investigation.

To further test our hypotheses, we used a selection of bacterial endophytes with different plant-adaptive traits and PGP properties (described in **Chapter 4**) to inoculate two soil types, allowing rice seeds to grown in these for up to five weeks (**Chapter 6**). To our surprise, the bacterial community from inside plant tissue (i.e. root and shoot) revealed minor differences between the inoculated and uninoculated treatments, whereas the rhizosphere and soil communities largely differed. Phylogenetic analyses of the endophytic communities revealed that more than 50% of the excised DGGE bands were similar (99.0% cut-off level) to those of the endophytic community assessed from mature rice plants growing in the paddy field in the Philippines. Further investigation showed that the rice seeds were an important source of bacterial endophytes observed in our experiment (**Chapter 6**). Moreover, using a direct molecular approach on the basis of directly extracted DNA, we observed that approximately 45% of the bacterial community from the first seed generation was again found in the second generation. This suggested that many selected bacteria are highly adapted to the plant environment. Considering the aforementioned synergistic interactions, I speculate that seed-borne bacteria are true competent endophytes and they may assist in the establishment of a new plant generation. Furthermore, we observed that several seed-borne bacterial endophytes were capable of colonizing the rhizosphere and even the soil surrounding the plant roots, indicating that endophytes might also be released from plant tissues. This might have a profound effect on the soil community structure, as the selected bacteria might alter the soil niche (e.g. pH, production of antibiotics) to the benefit of its own growth. While it is too early to weigh the importance of this observation, it is valid as a 'proof of concept'. However, one should bear in mind that our results were obtained under reduced microbial complexities and might differ under field conditions when microbial competition is harsh.

In **Chapter 7**, we investigated the role of bacterial endophytes that reside inside rice roots by direct metagenomic analysis. For habitat-specific fingerprints, quantitative gene content analyses were compared in several different metagenomes based on the relative abundances of protein-encoding genes. The rice endophyte metabiome was shown to differ from other investigated metabiomes (**Chapter 1**), suggesting the endosphere is a unique environment and not a subsample of the soil microbial community (as previously thought). Furthermore, the endosphere community showed, on average, a high genome size (estimated to be about 5.7 Mb). This is typical for communities dwelling in environments with a high diversity and fluctuating amounts of nutrient sources (Raes et al., 2007). Such environments may sustain bacteria with large genomes, also allowing highly complex

interactions to evolve. Abundant features found in the rice endophytic community included many plant-adaptive traits, while features potentially involved in plant-growth promotion were found in relatively low abundance. Given the complexity of the system, one might speculate that genes involved in plant-growth-promotion have been underestimated. Certainly, a more comprehensive metagenome analysis should be performed. Nevertheless, we present the first functional metagenome of the endosphere, in which many challenges were surpassed and new concepts were established. Hopefully, forthcoming studies will take into account the complexity of the system.

In this thesis, we learned that bacterial endophytes of rice represent a distinct community which differs from that found in soil. The endophytes are armed with many features that allow them to colonize the hostile plant tissues. Due to their metabolic versatility, bacterial endophytes might complement and/or impact on plant physiology, in particular cases improving plant growth. In this study, I present a glimpse of how important bacterial endophytes are as effectors of processes important for the plant life cycle. Understanding the functions and mechanisms of bacterial endophyte that play a role in their establishment in the endosphere will certainly improve agricultural management practices for a sustainable crop production.

Prospect for future research

Plants, as they grow, synthesise a vast number of photoassimilated compounds. It is assumed that many of the metabolic products are exuded via root tissue, which has a direct influence on the microbiological processes surrounding the plant. Hence, plants actively select bacteria, beneficial or not, and establish relationships with these. It has been stated that, in this process, detrimental interactions are repelled (Bais, 2006). Here, we show that this is not the only facet of the plant-bacterium interaction. Endophytes are also transmitted via seeds, which are thus capable of dissemination and propagation in and out of the newly-born plant generation. In addition, particular bacteria that occur as endophytes may even be released into the surrounding environment, thus modulating the local microflora, with potential implications for further plant development.

The observations presented in this thesis have a strong impact on the ecology of both microorganisms and plants. As shown, seed-borne bacterial endophytes are capable of colonizing the tissues of new plant generations and the surrounding soils. Therefore, one can assume that selected endophytes also directly influence the soil community, as the

presence of certain bacterial species (e.g. *Pseudomonas* spp., *Bacillus* spp.) is also likely to alter the soil microbial community structure (Garbeva, 2005). Furthermore, competent endophytes might also allow invasive plants to outcompete native plant species, thus increasing their fitness and dissemination capacities (Klironomos, 2002).

It is early to assume, but I dare to say that in virtually each stage of plant growth and development there is the need for one or many bacteria with specific characteristics that suite the host plants. For instance, seed germination is often enhanced by CK-producing bacteria (Holland & Polacco, 1994). At the seed premilk stage, introduction of *Pantoea agglomerans* strain YS19 enhanced, significantly, the transportation of the photosynthetic assimilation product from the flag leaves to the seeds (Feng et al., 2006). It is striking that many of these bacteria are indigenous to the host seeds but their function is often enhanced when inoculated, which hints at the importance of the number of bacterial cells to population functioning. Understanding plant physiology and how it affects the endophytic populations in each stage of host growth will secure food production for generations to come in a sustainable way, thus leading to the second "green revolution".

The study of bacterial endophytes is relatively new, as no more than twenty studies had been published annually in the years before 1995. Currently, new enthusiastic adepts (like myself) are contributing to foster our knowledge on this challenging area of research. There is a long path to be unravelled, but much can be learned from better-developed areas (e.g. plant pathology). And, the future is bright, as new findings will certainly hold promise for a more environmentally-friendly agricultural production with the use of endophytes.

Korte samenvatting

Landplanten hebben een lange geschiedenis van interacties met micro-organismen. In feite wordt gedacht dat primitieve planten zich op land konden vestigen na het aangaan van symbiose met arbusculaire mycorrhiza schimmels, die hen hielpen met het verkrijgen van water en voedingsstoffen uit de grond (Harrison, 2005). Plant-bacterie symbioses gaan verder terug in de tijd en staan aan de wieg van ons bestaan (Cavalier-Smith, 2010). Vandaag de dag zijn we ons ervan bewust dat plant-bacterie symbioses inherent zijn aan alle plantsoorten op aarde en dat interacties tussen planten en micro-organismen eerder regel dan uitzondering zijn. Daarom heeft het onderzoek beschreven in dit proefschrift als doel te identificeren wat de adaptieve en 'plant-beneficial' mechanismen zijn voor de 'establishment' van wederzijds voordelige relaties. Een uitgebreide discussie over hoe planten voordeel kunnen verkrijgen uit de met hen geassocieerde bacteriën wordt gegeven in **Hoofdstuk 1**, terwijl in **Hoofdstuk 2** wordt behandeld wat er nodig is voor een bacterie om een competente endofiet te worden. Er bestaan drie mechanismen die ervoor kunnen zorgen dat plant-endofiet interacties een win-win relatie vormen, waarin gastheerplanten voordeel halen uit groei en de geassocieerde endofieten voordeel hebben van een betrouwbare voedingsstoffenbron.

- Holland (1997) stelde voor dat het phytohormoon cytokinine vaak geproduceerd wordt door microbiële symbionten om plantgroei te stimuleren. Als consequentie van de groei worden sommige overtollige metabolieten gegenereerd en gebruikt door de bacteriën voor hun eigen groei. Productie van indool-3-azijnzuur (IAA) and cytokinines door endofytische bacteriën induceert vaak plantgroei. Om nieuw weefsel te vormen worden celwanden afgebroken waardoor methanol en andere producten vrijkomen. Het is daarom dat methylotrofe bacteriën en vele andere 'afvalgebruikers' plantgroei induceren waardoor deze metabolieten vrijkomen en hun eigen groei wordt verbeterd.

- Glick en collega's (1998) beschreven een model waarin rhizosfeerbacteriën die het enzym ACC deaminase bevatten plantgroei bevorderen door modulatie van de ethyleenconcentraties in de plant, waardoor de gevolgen van omgevinggeïnduceerde stress worden getemperd. In **Hoofdstuk 2** breid ik deze functie van rhizosfeerbewoners uit naar endofytische bacteriën. Onder stressvolle omstandigheden worden gastheergenen betrokken bij de synthese van ethyleen geïnduceerd en de

directe precursor van ethyleen, ACC wordt uitgescheiden door de gastheercellen om omgezet te worden in ethyleen. In de aanwezigheid van ACC deaminase bevattende bacteriën wordt de ACC opgenomen en gebruikt als koolstofbron (ACC → NH3 + α-ketobutyrate), waardoor de hoeveelheid geproduceerde ethyleen wordt verminderd.

- Taghavi en collega's (2010) hebben kortgeleden aangetoond hoe *Enterobacter* sp. 638 de groei van populieren kan stimuleren. Door het gehele genoom van deze bacterie te sequencen vonden de auteurs een genomische regio met genen betrokken bij plantgroeistimulatie en sucrosemetabolisme. Transcriptomische en metabolische analyses tonen aan dat de synthese van VOCs acetoin and 2,3-butanediol werd geïnduceerd door de opname van sucrose. Deze VOCs worden beschouwd als fytohormonen die betrokken zijn bij plantgroeistimulatie en tevens gerelateerd zijn aan de inductie van systemische weerstand. Derhalve begunstigt de opname van sucrose de bacteriële groei en de productie van VOCs, die op hun beurt de groei van de gastheerplant bevorderen.

Wanneer planten groeien, synthetiseren ze een groot aantal verbindingen. Er wordt aangenomen dat de meeste metabole producten via wortelweefsel worden uitgescheiden, een proces dat direct invloed heeft op de biologische processen die plaatsvinden in de omgeving van de plant. In het licht van de nauwe samenhang tussen plantfysiologie en de samenstelling van de plant-geassocieerde bacteriële gemeenschappen, stel ik voor dat de effecten van deze factoren een belangrijke factor vormen voor de plantgeassocieerde en, met name, de endofytische bacteriële gemeenschappen. Uitgaande van deze hypothese, hebben wij de effecten van plant genotype, bodemsoort en de efficiëntie van nutrientengebruik op de samenstelling van verschillende bacteriële gemeenschappen geassocieerd met de wortelweefsels van tien rijstrassen onderzocht (**hoofdstuk 3**). Eeen significante correlatie van het rijst genotype (ras) met de samenstelling van zowel de totale bacteriele gemeenschap alsook de *Alpha-* en *Beta-proteobacteria* en *Pseudomonas* werd waargenomen. Slechts in een enkel geval, met name betreffende de *Actinobacteria*, was er een betere correlatie met bodemsoort. Interessant was dat de rassen van *Oryza sativa* ondersoort *indica* een trend vertoonden om vergelijkbare bacteriele gemeenschappen te selecteren, terwijl de rassen van de ondersoort *japonica* en *aromatica* selecteerden voor afwijkende bacteriele gemeenschappen. Dit verschijnsel is naar alle waarschijnlijkheid toe te schrijven aan de verschillen in fysiologische eigenschappen van de plant, gedicteerd door haar genetische 'make-up'. *Indica* rassen bijvoorbeeld zijn intensief veredeld om

hoge opbrengsten te bewerkstelligen (hetgeen hun genetische variëteit gereduceerd kan hebben), terwijl *japonica* rassen een grote genetische variatie hebben behouden voor rijstproductie onder zware milieuomstandigheden. Het lijkt erop dat de twee strategieën geresulteerd hebben in verschillende bacteriële gemeenschappen.

Een van de eerder onderzochte rijstrassen, t.w. APO, is in het onderzoek verder gebruikt om de endofytische bacteriele gemeenschappen van de rijstwortel te onderzoeken met kweekafhankelijke en -onafhankelijke methoden (**Hoofdstuk 4**). Fylogenetische analyses van deze gemeenschappen met behulp van op het 16S rRNA gen gebaseerde bibliotheken toonden een aanzienlijke diversiteit in de prokaryotische gemeenschap aan, met 16 fyla/klassen, terwijl de gemeenschap verkregen via isolatie beperkt was tot 5 klassen, maar overeenkomende met de direct verkregen sequenties en ongeveer 33% vertegenwoordigde. Voor zover wij weten geeft onze studie het tot dusverre meest complete overzicht van ongecultiveerde en gecultiveerde endofytische bacteriën van rijst, waardoor de lijst van bekende rijst endofieten verder wordt uitgebreid. *Gammaproteobacteria* vormden verreweg de grootste klasse, met leden van *Enterobacter* als de meest frequente in beide methoden. Analyses van bacteriele plant-'adaptive' en plantgroeistimulerende eigenschappen toonden aan dat veel van de geselecteerde bacteriestammen meer dan 1 activiteit vertoonde, wat aangeeft dat endofieten sterk aangepaste bacterien zijn die gedijen binnen de gastheerplant om hun groei te bevorderen. Daarnaast gaven de resultaten van de koolstofbronverbruikprofielen aan dat verschillende trofische niveaus in een microbiele gemeenschap kunnen co-existeren, hetgeen misschien ook de verbazingwekkende bacteriele diversiteit binnen de rijstwortels verklaard.

Verdere karakterisering van de meest frequent voorkomende *Enterobacter*-gerelateerde stammen leidde tot the identificatie van twee nieuwe soorten met als voorgestelde namen *Enterobacter oryziphylus* stam REICA_142^T en *Enterobacter oryzendophyticus* stam REICA_082^T (**Hoofdstuk 5**). Beide stammen vertoonden een surplus aan plantgroeistimulerende eigenschappen en waren relatief nauw verwant op het niveau van de 16S rRNA en *rpoB* genen aan andere plantgroeistimulerende *Enterobacter* soorten. De introductie van *E. oryziphilus* strain REICA_142^T in rijst vertoonde een (niet significante) toename van 35% in plantversgewicht in niet-steriele grond. *Enterobacter* zou de groei van planten kunnen stimuleren via verschillende mechanismen, maar onze stam vertoonde *in vitro* een hoge stikstofbindingsactiviteit.

Om onze hypothesen verder te toetsen hebben we gebruikgemaakt van een selectie van bacteriële endofieten met verschillende 'plant-adaptive' en plantgroeistimulerende

eigenschappen (beschreven in **Hoofdstuk 4**). Twee bodemsoorten werden beent en rijst werd erop gecultiveerd gedurende 5 weken (**Hoofdstuk 6**). Tot onze verrassing vertoonde de bacteriële gemeenschap in de plant (wortel en stam) slechts geringe verschillen tussen de beënte en niet-beënte behandelingen, terwijl de rhizosfeer- en bodemgemeenschappen sterk verschilden. Verder onderzoek liet zien dat rijstzaad een belangrijke bron van bacteriële endofieten was. Een moleculaire aanpak op basis van direct geëxtraheerd DNA toonde aan dat ongeveer 45% van de bacteriële gemeenschap van de eerste zaadgeneratie kon worden teruggevonden in de tweede generatie. Dit suggereert dat vele geselecteerde bacteriën sterk aangepast zijn aan de plantomgeving. In overeenstemming met de hierboven genoemde synergistische interactie, speculeer ik dat bacteriën uit rijstzaad zeer competente endofieten zijn die nieuwe plantgeneraties helpen zich te vestigen. Daarnaast hebben we waargenomen dat verschillende bacteriële endofieten afkomstig van zaad in staat waren de rhizosfeer en zelfs de bodem rondom de wortels te koloniseren, waarmee aangegeven wordt dat endofieten misschien wel vrijkomen vanuit de plant. Hoewel het te vroeg is om de werkelijke waarde van deze observaties aan te geven, zijn zij van belang als 'proof of concept'.

In **Hoofdstuk 7** onderzoeken we de rol van bacteriële endofieten die zich vestigen in de rijstwortels door middel van directe metagenomische analyses. Teneinde habitat-specifieke 'fingerprints' te verkrijgen, werden kwantitatieve gen 'content' analyses vergeleken in verschillende metagenomen gebaseerd op de relatieve aanwezigheid van genen ingedeeld in klassen. Het 'metabioom' van rijstendofieten verschilde van andere onderzochte metabiomen waardoor het lijkt alsof de endosfeer een unieke omgeving is en niet een deelverzameling van de microbiële bodemgemeenschap zoals voorheen werd gedacht. Daarnaast vertoonde de endosfeergemeenschap een gemiddelde genoomgrootte die geschat werd op 5.7 Mb. Een dergelijk groot genoom is typisch voor microbiele gemeenschappen die leven in omstandigheden met hoge aanwezigheid en diversiteit aan nutriëntbronnen. Deze rijke en soms wisselende typen en hoeveelheden nutrienten onderhouden micro-organismen met grote genomen, waartussen ook complexe interacties tot ontwikkeling kunnen komen. Wijdverbreid in de rijst endofytische gemeenschap waren veel 'plant-adaptive' eigenschappen, terwijl de eigenschappen die potentieel betrokken zijn bij plantgroeipromotie in veel lagere hoeveelheden aanwezig waren.

Dir proefschrift heeft ons geleerd dat bacteriele endofieten van rijst een gemeenschap vormen die zich onderscheidt van de gemeenschap die in de bodem aangetroffen wordt. De endofieten zijn uitgerust met veel verschillende eigenschappen die de kolonisatie van

plantweefsel mogelijk maken. Door hun metabolische verscheidenheid zouden bacteriële endofieten de plantfysiology kunnen complementeren / beïnvloeden, hetgeen in bepaalde gevallen plantgroeiverbeterend kan werken. Echter, aangezien endofieten direct afhankelijk zijn van door de plant geproduceerde metabolische verbindingen zullen omgevingsfactoren die de plantfysiologie en daardoor de plant-metabolische processen beïnvloeden, ook invloed hebben op de endofytische gemeenschappen. In deze studie presenteer ik de eerste glimp van het belang van bacteriele endofytische gemeenschappen als effectoren van belangrijke levenscyclusprocessen van planten. Het begrijpen van de functies en mechanismen van bacteriele endofieten die een rol spelen in hun vestiging in de endosfeer zullen zeker leiden tot de verbetering van agrarische beheerpraktijken die van belang zijn voor het bevorderen van een duurzame teelt.

Vooruitzicht voor toekomstig onderzoek

Planten synthetiseren een groot aantal ´foto-geassimileerd´verbindingen wanneer ze groeien. Veel van de metabolische producten worden via de wortel uitgescheiden, wat een direct effect heeft op de microbiele processen rond de plant. Dus, planten selecteren actief bacteriën die heilzaam zijn of juist niet en gaan relaties aan met hen. Er wordt wel verondersteld dat, in dit proces, de voor de plant nadelige interacties worden gedeselecteerd (Bais, 2006). In deze studie laten we zien dat dit niet het enige belangrijke facet van de plant-bacterie interacties is. Bacteriele endofyten worden ook doorgegeven via zaden, leidend tot verspreiding naar en vermeerdering in de nieuwe plantgeneraties. Daarnaast kunnen bepaalde bacteriën die voorkomen als endofieten zelfs worden uitgescheiden naar de omgeving, derhalve de plaatselijke microflora modulerend, met mogelijke gevolgen voor de verdere ontwikkeling van de plant.

De waarnemingen die in dit proefschrift worden gepresenteerd hebben een sterke invloed op het denken over de ecologie van zowel micro-organismen als de plant. Zoals aangetoond, zijn de zaadoverdraagbare bacteriële endofieten in staat tot kolonisatie van de weefsels van nieuwe plantgeneraties en de omliggende bodem. Daarnaast mag men aannemen dat de door de plant geselecteerde endofieten ook direct de bodemgemeenschap beinvloeden, aangezien de aanwezigheid van bepaalde bacteriesoorten (bv. *Pseudomonas* spp., *Bacillus* spp.) de structuur van de microbiële gemeenschap in de bodem kan veranderen (Garbeva, 2005). Bovendien zouden competente endofieten ook een rol kunnen spelen bij de vestiging van invasieve planten, hetgeen inheemse plantsoorten negatief

beinvloedt. De invloed van de endofieten zou de conditie en verspreidingscapaciteiten van de invasieve planten kunnen doen toenemen (Klironomos, 2002).

Het is te vroeg om dit met zekerheid te stellen, maar ik durf te poneren dat in ieder stadium van plantgroei er een behoefte is aan een of meerdere bacteriën met specifieke eigenschappen die bij de gastheerplant passen. Zaadkieming bijvoorbeeld wordt vaak gestimuleerd door cytokinine-producerende bacteriën (Holland & Polacco, 1994) en enting met *Pantoea agglomerans* stam YS19 tijdens het zaad premilk stadium verbeterde het transport van fotosynthetaten van de bron (blad) naar de bestemming (zaad) (Feng et al., 2006). Het is verbazingwekkend dat veel van deze bacteriën waarschijnlijk inheems zijn in zaad, maar dat hun functie vaak wordt versterkt wanneer ze geïntroduceerd worden. Dit geeft aan dat de aantallen (dichtheid) bacteriële cellen belangrijk zijn voor het functioneren van populaties. Het begrijpen van de plantfysiologie, hoe deze de endofytische populaties beïnvloedt en hoe, in retributie, endofyten de plant assisteren in vestiging, groei en productie, kan leiden tot een tweede groene revolutie, waardoor voedselproductie voor de komende generaties op een duurzamer wijze tot stand kan komen.

Het bestuderen van bacteriële endofieten is een relatief jong vakgebied, met minder dan 20 studies gepubliceerd tot 1995. Het huidige onderzoek, gestimuleerd door de nieuwe geavanceerde technieken, draagt in hoge mate bij aan onze kennis over dit interessante onderzoeksgebied. Er is nog een lange weg te gaan, maar veel kan worden geleerd van de kennis in de verder ontwikkelde gebieden (zoals plantpathologie en microbiële bodemgemeenschapen). Voor wat betreft toepasbaarheid voorzie ik een goede toekomst voor het endofytonderzoek, bijdragend aan een milieuvriendelijker landbouw waarin het gebruik van endofieten centraal staat.

Resumo

Plantas terrestres e micro-organisms formam associações de longo período. De fato, tem sido especulado que plantas primitivas só foram capazes de colonizar ambientes terrestres após estabelecer simbiose com fungo micorrízico arbuscular, o qual fornecia água e nutrientes provenientes do solo para a planta hospedeira (Harrison, 2005). Simbioses entre plantas e bactérias são ainda mais primitivas e estão na origem da nossa existência (Cavalier-Smith, 2010). Hoje, sabemos que simbioses entre plantas e micro-organisms são essenciais para cada espécie de hospedeiro e que as interações estabelecidas são mais comuns do que antes se imaginava. Por esse motivo, as pesquisas realizadas nesse manuscrito procuraram identificar mecanismos usados por bactérias endofíticas no processo de adaptação e nos benefícios decorrentes dessas interações para a planta hospedeira de arroz. Uma discussão detalhada de como plantas beneficiam-se das bactérias associadas a elas é apresentada no **Capítulo 1**, entretanto no **Capítulo 2** nós discutimos o que é necessário para uma bactéria tornar-se competente dentro da planta hospedeira (endofítica). Essas interações afetam principalmente a fisiologia da planta através da produção e do controle de fitohormônios. Aqui, retratamos, pelo menos, três mecanismos que exemplificam como interações sinergísticas entre planta e bactérias endofíticas podem tornar uma relação de ganho para ambos:

- Holland (1997) propôs que o fitohôrmonio citocinina é produzido por micro-organismos simbiontes para estimular o crescimento da planta hospedeira e como consequência do crescimento da planta, alguns compostos metabólicos são liberados e usados pelas bactérias para o seu próprio crescimento. Por exemplo, a produção de citocinina e auxinas por bactérias endofíticas geralmente induzem o crescimento das plantas. Durante a formação de novos tecidos, ocorre a degradação da parede celular, liberando metanol e outros compostos. Desta forma, bactérias metilotróficas e muitas outras oportunistas podem induzir o crescimento da planta para obter os metabólicos necessários para o seu próprio crescimento. Esse tipo de interação é favorecida principalmente durante o crescimento inicial da planta, onde existe uma constante formação de tecidos novos.

- Glick et al. (1998) descreveram um modelo no qual bactérias da rizosfera capazes de sintetizar a enzima ACC deaminase são responsáveis por promover o crescimento de plantas hospedeiras através do controle do nível de produção de

etileno, reduzindo com isso o estresse induzido por fatores ambientais. No **Capítulo 2**, esse mequanismo foi estendido para as bactérias endofíticas. Em condições desvaforávies, genes da planta envolvidos na síntese de etileno são ativados e o precursor imediato de etileno, ACC é produzido e secretado pelas células da planta para depois ser convertidos em etileno. Bactérias contendo ACC deaminase rapidamente incorporam ACC, evitando a formação de etileno, que por sua vez reduzo efeito prolongado dos estresses ambientais.

- Recentemente, Taghavi et al. (2010) demonstraram como a bactéria *Enterobacter* sp. 638 estimula o crescimento das árvores hospedeiras de álamo. Após o sequenciamento do genoma completo de *Enterobacter* sp. 638, os autores encontraram uma região genômica responsável pela síntese de compostos orgânicos voláteis (COVs - acetoína e 2,3-butanodiol) e metabolismo de sucrose. Ambos COVs são fitohormônios envolvidos no crescimento das plantas e também na indução de resistência sistêmica. Mais ainda, análises do transcriptoma e metaboloma revelaram uma correlação positiva entre a produção de COVs e a aquisição de sucrose, um dos principais açúcares produzidos por árvores de álamo.

Durante o crescimento das plantas, vários compostos são sintetizados. É consenso dizer que a maioria dos produtos metabólicos sintetizados são secretados através dos tecidos das raízes, os quais afetam diretamente os processos biológicos que ocorrem ao redor das plantas. Considerando que existe uma conecção direta entre a fisiologia da planta e a estrutura das comunidades bacterianas associadas à planta, foi especulado nesse estudo que o efeito desses fatores afetam a comunidade de ambas, bactérias associadas e bactérias endofíticas igualmente. No **Capítulo 3**, nós investigamos os efeitos do genótipo da planta, tipo de solo e a eficiência no uso de nutrientes pela planta sobre a composição de diferentes comunidades bacterianas associadas aos tecidos das raízes de dez cultivares de arroz. Uma correlação significativa foi observada entre genótipos de arroz e a composição de todas as bactérias, assim como para as classes específicas de *Alfa-*, *Beta-proteobacteria* e *Pseudomonas*. Em um único caso, a comunidade de *Actinobacteria* correlacionou melhor com o tipo de solo. Interessante que cultivares de *Oryza sativa* sub-espécie *indica* selecionam comunidades bacterianas similares entre si, enquanto cultivares pertencentes à sub-espécies *japonica* and *aromatica* selecionam communidades que divergem entre si. Essa observação pode ser explicada pelas diferenças oriúndas das características fisiológicas, as quais são determinadas pela interação genótipo-fenótipo e específica de

cada planta hospedeira. Exemplificando, cultivares de arroz originados de plantas *indica* tem sido amplamente melhorados para obter altas produções, embora cultivares originados de plantas *japonica* são principalmente melhorados visando uma alta variabilidade genética que optimiza a produção de grãos em condições ambientais desvaforáveis. Dessa maneira, não é surpressa que ambas estratégias de melhoramento resultam na associação de communidades bacterianas distintas.

Um cultivar de arroz, denominado APO, foi novamente usado para avaliar a comunidade endofítica das raízes através do uso de técnicas de cultivo-dependente e -independente (**Capítulo 4**). Analíses filogenéticas do gene ribossômico de 16S RNA oriundos de biblioteca de clones revelaram uma grande diversidade na comunidade de micro-organismos procariontes, compreendendo ao todo 16 filos/classes, enquanto a comunidade assessada por cultivo limitou-se a 5 classes de bactérias. Porém, a comunidade bacteriana isolada representou cerca de 33% da comunidade observada pelos clones. No melhor do nosso conhecimento, essa investigação é a mais abrangente análise realizada com endofíticos de arroz, revelando a presença de classes de micro-organismos jamais identificadas em arroz. Em ambas abordagens, membros de *Gammaproteobacteria* foram de longe os mais assessados, e deste o gênero *Enterobacter* o mais abundante. Análises das propriedades fisiológicas mostraram que todas as espécies investigadas possuem mais de uma propriedade potencialmente envolvidas na adaptação e na promoção de crescimento da planta, sugerindo que essas bactérias endofíticas são altamente adaptadas para habitarem os tecidos internos da planta assim como de promover o crescimento da mesma. Mais ainda, os resultados obtidos provenientes da utilização de diversas fontes de carbonos indicaram que bactérias endofíticas possuem vários níveis tróficos de alimentação, explicando desta forma a alta diversidade observada dentro das raízes de arroz.

Seis estirpes de *Enterobacter* representando as mais abundantes foram posteriormente usadas para estudo de classificação taxonômica. Essa análise resultou na identificação de duas novas espécies, as quais foram propostos os nomes *Enterobacter oryziphilus* strain REICA_142^T and *Enterobacter oryzendophyticus* strain REICA_082^T (**Capítulo 5**). Ambas estirpes revelaram várias propriedades capazes de promover o crescimento das plantas hospedeiras e foram encontradas próximas, ao nível dos genes 16S e *rpoB* com outras espécies de *Enterobacter* que apresentam propriedades similares. Inoculação de *E. oryziphilus* strain REICA_142^T em sementes de arroz revelaram um aumento, embora não significativo, de 35% no ganho de matéria fresca produzida quando as plantas foram cultivadas em solo não estéril por um período de cinco semanas. Entre as propriedades

benéficas para a planta, nossas estirpes revelaram alto potencial para fixação de nitrogênio atmosférico, em condições *in vitro*.

Para testar uma das hipóteses formuladas nessa tese, nós usamos uma seleção de bactérias endofíticas com distinto níveis de interação com a planta, além de diferentes propriedades para promover o crescimento da planta hospedeira. Sementes de arroz foram introduzidas em dois tipos de solos previamente inoculados com as bactérias (**Capítulo 6**). Para nossa surpresa, a comunidade bacteriana assessada de dentro dos tecidos das plantas (raízes e caule) revelaram pouca diferença entre os tratamentos com e sem inóculo, entretanto as comunidades da rizosfera e do solo foram diferentes. Novas investigações mostraram que, nas condições do nosso experimento, as sementes de arroz foram uma fonte importante de bactérias endofíticas. Mais ainda, análises moleculares provenientes do DNA genômico extraído diretamente das sementes de arroz revelaram que aproximadamente 45% da comunidade bacteriana presente na primeira geração de sementes foi encontrada novamente na segunda geração, sugerindo que communidades específicas de bactérias são altamente adaptadas ao ambiente da planta hospedeira. Considerando as interações sinergistícas mencionadas acima, assume-se que as bactérias oriundas das sementes são verdadeiramente endofíticas competentes, as quais podem auxiliar na colonização de novas áreas por planta invasora. Além disso, nós observamos que muitas das bactérias endofíticas provenientes das sementes foram capazes de colonizar a rizosfera e até mesmo o solo ao redor das raízes, indicando que essas bactérias podem ser também liberadas dos tecidos da planta. Entretanto ainda é cedo para visualizar a importância dessa observação para a ciência do solo, porém a mesma é válida com uma "prova de conceito" de que communidade de bactérias endofíticas são provenientes de ambos semente e solo.

No **capítulo 7**, nós investigamos a função das bactérias endofíticas residentes no interior das raízes de arroz com o uso de análise metagenômica. Para obter características específicas do ambiente, análise quantitativa de cada gene foi comparada com diferentes estudos metagenômicos baseando-se na abundância relativa dos genes envolvidos na codificação de proteínas. O metabioma de bactérias endofíticas do arroz diferencia-se de outros metabiomas investigados, sugerindo que os tecidos internos da planta formam um habitat único e não somente uma sub-amostra da comunidade microbiana do solo como antes pensava-se. Além disso, a comunidade bacteriana endofítica possui em média alto conteúdo genômico (estimado em 5.7 Milhões de pares de base). Essas comunidades são típicas de ambientes ricos em diversidade assim como em abundância de fontes de

nutrientes, os quais permitem sustentar comunidades com alto conteúdo genômico e também com alta complexidade de interações (Raes et al., 2007). Como havíamos especulado, as características mais abundantes encontradas na comunidade endofítica de arroz incluem vários propriedades envolvidas na adaptação ao ambiente interno da planta hospedeira. As características potencialmente envolvidas na promoção do crescimento da planta também foram encontradas, porém em relativa baixa abundância.

Nesta tese, nós aprendemos que bactérias endofíticas do arroz pertencem a uma comunidade distinta das outras encontradas no solo. Os endofíticos possuem várias características que permitem a colonização dos tecidos inóspitos da planta. Devido a versatilidade metabólica, as bactérias endofíticas podem complementar ou alterar a fisiologia da planta hospedeira, em casos benéficos promovendo o crescimento das plantas. Neste estudo, as primeiras vertentes da importância das bactérias endofíticas como promotores dos processos essenciais para o ciclo de vida das plantas são apresentadas. Entender as funções e mecanismos usados pelas bactérias endofíticas durante o processo de estabelecimento certamente irá melhorar as práticas de manejo na agricultura levando a uma produtividade mais sustentável e harmoniosa com o meio-ambiente.

Prospectos para pesquisas futuras

Plantas, durante o crescimento, sintetizam um vasto número de compostos foto assimilados. É de comum senso que muitos dos produtos metabólicos são exudados através dos tecidos das raízes, os quais exercem direta influência nos processos microbiológicos que acontecem ao redor das plantas. Assim, plantas ativamente selecionam bactérias, benéficas ou não, para estabelecer relações. Tem sido proposto que, durante esse processo, interações prejudiciais são repelidas (Bais, 2006). Aqui, nós mostramos que este conceito não é a única faceta da interação planta-bactéria. Bactérias endofíticas também são transmitidas por semente, e são capazes de disseminação e propagação tanto dentro quanto fora da recente formada plantícula. Além disso, algumas bactérias originalmente endofíticas podem até ser liberadas para o ambiente ao redor das raízes, desta forma alterando a comunidade microbiana local, e levando a potenciais implicações para o desenvolvimento posterior das plantas hospedeiras (Puentes et al., 2009).

As observações apresentadas nessa tese têm um forte impacto na ecologia de ambos, plantas e micro-organismos. Como demonstrado, bactérias endofíticas originadas das sementes são capazes de colonizar os tecidos das novas plantículas assim como o solo adjacente. Portanto, alguém pode assumir que algumas bactérias endofíticas também

possam influenciar diretamente a comunidade do solo, como demonstrado anteriormente por certas espécies de bactérias (ex: *Pseudomonas* spp., *Bacillus* spp.) (Garbeva, 2005). Além disso, bactérias endofíticas competentes podem também permitir que plantas invasoras tenham vantagens competitivas sobre as espécies nativas, desta forma aumentando sua sobrevivência e capacidade de disseminação (Klironomos, 2002).

Ainda é cedo para advogar, mas arrisco em dizer que em cada fase do crescimento e desenvolvimento da planta existe a necessidade de uma ou várias bactérias com características específicas que atendam as exigências fisiológicas das plantas hospedeiras. Por exemplo, a taxa de germinação das sementes é frequentemente aumentada na presença de bactérias produtoras de citocininas (Holland & Polacco, 1994), durante a fase de maturação de grãos, inoculação da estirpe YS19 de *Pantoea agglomerans*, aumenta significativamente a translocação de produtos foto-assimilados provenientes das folhas para as sementes (Feng et al., 2006). É interessante notar que, muitas das bactérias encontradas nas sementes tem sua função benéfica ampliada somente quando inoculadas, o que leva a suspeitar da importância do número de células bacterianas para ativar sua funcionalidade como um todo. Entender a fisiologia da planta e como essa afeta diretamente as populações de bactérias endofíticas poderá assegurar produção de alimento de forma sustentável para as gerações futuras, resultando na segunda "revolução verde".

Estudos sobre bactérias endofíticas são relativamente novos, com menos de 20 trabalhos publicados anualmente antes de 1995. Atualmente, adeptos entusiasmados estão contribuindo para o avanço de novos conhecimentos. Porém existe um longo caminho a ser trilhado, e muito pode ser aprendido através dos conhecimentos gerados em áreas mais desenvolvidas (ex. patologia de plantas, communidade microbiana do solo). O futuro é claro, novas descobertas com o uso de bactérias endofíticas certamente irão promover uma produção agrícola menos poluente e mais adequada ao equilíbrio harmonioso entre homem e o meio-ambiente.

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Acknowledgments

The research studies described in this manuscript were achieved with the contribution of many collaborators. First of all, I am gratefully for the opportunity given by Prof. **Jan Dirk van Elsas** to conduct my PhD study. He not just opens the doors of his lab, but also gave total freedom to conduct the studies as I whished. Most important of all, with a great deal of patiency, Dick correct over and over my English and in this way, I improved my writing skills. If the main fuction of the supervisor is to teach how to write a scientific manuscript, Dick should be placed among the greatest ones. I'm also gratefully for **Leo van Overbeek** to be my copromotor. Although many times we discussed on vary topics of my research project, sometimes I fought fearlessly to defend my ideas: "there are no oligotrophic endophytes", **Leo** always tried to bring the discussion to a higher level. His determination for high quality scientific manuscripts thought me to pay more attention to small details. He also contributed with practical experiences, besides his lab was always opened to conduct research.

Among the closest ones that walked with me in this journey was my wife, **Cristiane.** I will be unfair to myself if not dedicating here kind words of appreciation. Throghout my PhD studies, Cris gave daily contributions with long after-working hours on discussion the research progress, correcting manuscript drafts and given crutial ideas for the improvement of the study. I'm gratefully for this functional engagement.

I owed a great amount of gratitude to **Eelco Hoogwout** and **Özgül Inceoglu**. During winter and in many occasions with no place to go, I was provided with warm and love accommodation. I'm gratefully for their unconditional friendship and for the time we passed together mainly discussing ordinary subjects. My acknowledgement also goes to the essential contribution done by **Jolanda Brons** both in Philippines and in Netherlands, to **Fernando D. Andreote** for his contributions mainly in the first year of my studies, to **Rashid Nazir** for his expertise knowledge in microbial ecology and readiness contributions upon wide-ranging requests, to **Riitta Nissinen** for the constructive discussion on endophytes subject, to **Patricia Stevens, Michele Pereira e Silva, Alexander Semenov, Joana Salles, Cyrus Mallon, Stephanie Grizard, Silvia Cretoiu, Anna Kielak, Pilar Puentes, Rodrigo Costa** and **Tina Keller** for the friendly atmosphere created in- and out-of-the-lab, to the students **Jonas Bremer** and **Joras** for their contributions and to **Felipe Gomes**, **Vaso**

Lagou, **Fotini Karakassi**, **Anne Weiβ**, **Anouk Piquet**, **Evelien te Peole**, **Nuria Bonilla** and **Mario Chiurazzi** for their friendship.

Part of the studies presented in this manuscript were generated with the contribution of abroad collaborators, mainly **Angela Sessitsch** (Austrian Institute of Technology, Austria) for idealizing the metagenome project together with **Dick. Darshan Brar** (International Rice Research Institute, Philippines) for providing the rice materials, **Barbara Reinhold-Hurek** (University of Bremen, Germany) for leading the metagenome analysis and **Tanja Woyke** (Department of Energy, Joint Genome Institute, United States of America) for the bioinformatics analyses of the metagenome data. Also by those that were not direct involved in the project. My sincere gratitude to **Bea Scholten, Manon Dusseljee** and **Joukje Vries-Veldkamp** for office-related contributions, to **Louis Huisman** for microbiology-related assays, to **Muhammad** and **Anna** for cleaning most of our lab-generated mess.

I'm gratefully for the support and thoughts of encouragement from my parents, relatives and oversea friends.

The author